Improvement of Bioethanol Production

using *Saccharomyces cerevisiae*

A Thesis

Submitted to the College of Graduate Studies and Research

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the Department of Chemical Engineering

University of Saskatchewan

Saskatoon

By

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Acknowledgements

Several people need to be acknowledged for the successful completion of my Master of Science (M. Sc.) degree. First, I would like to express my gratitude and thanks to my supervisor, Dr. G. A. Hill. His guidance and advice has been invaluable. Second, I would like to thank my co-supervisors, Dr. C. Niu and Dr. W. J. Roesler for their constant support and guidance.

I would also like to thank my committee members, Dr. Yen-Han Lin and Dr. Hui Wang for their meaningful suggestions.

My gratitude goes to my parents. Without their faith in me I would not have made it this far.

My appreciation goes to Sandip for his support throughout the last few years. It is because of his understanding that I am able to complete my degree in a timely manner.

I would also like to thank Richard and Ted for their expert technical advice.

I am grateful to my lab mates Hossein and Pardeep who were always willing to answer my questions.

My gratitude goes to the Saskatchewan Agriculture Development Fund for their financial support for this project. I would also like to thank the College of Graduate Studies and Research for the Equity scholarship awarded to me in 2007. My appreciation also goes to BBI International from awarding me the 2008 Fuel Ethanol Workshop (FEW) scholarship.

Dedicated to my parents, Dimple Bawa and Surdev Bawa

and

to my sister, Navyata Bawa.

They are the strongest inspiration in my life.

Abstract

Ethanol, when mixed with gasoline, is an economical and environmentally friendly liquid fuel. Yeast cells under anaerobic conditions can ferment glucose to ethanol. However, glucose is expensive for industrial applications and starch is an economical alternative.

Simultaneous cold starch hydrolysis and fermentation was investigated for ethanol production from wheat starch particles. With a view to minimize process costs while maintaining a satisfactory ethanol yield, both a recombinant yeast cell and an inexpensive medium were tested for their fermentation abilities. Initially, NRRL Y132 strain was compared to Muntons yeast for their abilities to produce ethanol from glucose. Both the wild-type and the recombinant NRRL Y132 strains were cultured on soluble starch to determine if the plasmid bearing strain could produce ethanol without the addition of α-amylase. Finally, Muntons yeast was cultured on starch particles using both expensive and inexpensive media. Sequential hydrolysis and fermentation runs were performed using the inexpensive medium, with hydrolysis carried out at 30°C, 37.5°C, 45°C and 52.5°C.

The wild-type, NRRL Y132 strain grew faster and produced more ethanol than Muntons yeast when cultured on glucose. Compared to the wild-type strain, the recombinant NRRL Y132 strain did not show enhanced ethanol production from soluble starch. The results of the simultaneous hydrolysis and fermentation runs showed that the ethanol yields for runs performed in expensive medium (0.41, 0.38 and 0.42 g ethanol / g glucose) were slightly lower than those for runs performed in the inexpensive medium (0.46, 0.44 and 0.43 g ethanol / g glucose). The growth rates for the expensive and inexpensive media runs were comparable. Hence, it was concluded that the inexpensive medium can be used for ethanol production from starch particles with good ethanol productivities. For the sequential hydrolysis and fermentation runs, it was observed that the growth rates (0.11, 0.10, 0.10 and 0.11 h^{-1}) as well as the ethanol yields (0.44, 0.37, 0.44 and 0.39 g ethanol / g glucose) were similar in spite of the four different hydrolysis temperatures. Therefore, it was concluded that increasing the temperature above 30°C for enhancing starch particle hydrolysis does not increase fermentation productivity significantly.

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Nomenclature

Symbols

μ	specific growth rate
μ_m / μ_{max}	maximum specific growth rate
K_S	saturation constant

Abbreviations

GC	Gas Chromatography
FID	Flame Ionization Detector
OD	Optical Density

1.0 Introduction

1.1 Bioethanol as a Sustainable Fuel

An efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases (Altıntaş *et al*., 2002). Yeast cells (*Saccharomyces cerevisiae*) are facultative anaerobes and under anaerobic conditions can ferment glucose to ethanol. *S. cerevisiae* is ideal for ethanol production due to several properties including fast growth rates, efficient glucose repression, efficient ethanol production and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels. Glucose is broken down to form pyruvate in most organisms via the glycolytic pathway and this pyruvate can result in the production of ethanol under anaerobic conditions. The energy for growth of cells during ethanol production is provided by the glycolytic and fermentation pathways (Pisˇkur *et al*., 2006). In North America, glucose is an expensive raw material for industrial applications and therefore starch is an economical alternative. Approximately 25 million tons of wheat are grown every year in Canada, 50% of which is produced in Saskatchewan (Textor *et al.*, 1998).

This work deals with the production of bioethanol from wheat starch, but it is important to be aware of some prevailing issues in society regarding the 'food versus fuel' debate. There are two major categories of biomass that are used for biofuel production. The first category is crops and grains like corn, wheat, sugarcane, soybeans, etc. and the second category contains waste biomass such as straw, corn stover and waste wood (Tilman *et al*., 2006). The second category is much more inexpensive and because it is a waste material, it is more ethical to use for bioethanol production as compared to the first category. According to one point of view, today, due to increased demands for both energy and food, there are concerns regarding the production of food-based bioethanol. Biofuel production also competes with food production with regard to fertile land available. On the other hand, an opposite view-point states that we do not require choosing between fueling our cars and feeding people because we can do both effectively (Sneller and Durante, 2007). Biofuels are not only promising sources of environment-friendly energy, but also provide an economic opportunity for the agriculture industry worldwide. In 2005 the world ethanol production was approximately 46 billion litres per year, which is

expected to reach 76 billion litres per year by 2010 (Olfert and Weseen, 2007). Table 1.1 shows world ethanol production country-wise.

Table 1.1: World Ethanol Production by Country – 2005

Country	Production (millions of litres)	Country	Production (millions of litres)
United States	16,139	South Africa	390
Brazil	15,999	Spain	352
China	3,800	United Kingdom	348
India	1,699	Thailand	299
France	908	Ukraine	246
Russia	749	Canada	231
Germany	431	Others	1,707

Source: The Saskatchewan Institute of Public Policy, Paper 48

Bioethanol, in particular has great potential as a renewable, non-toxic and clean alternative fuel, thereby reducing dependence on fossil energy. Fermentation of sugars derived from energy crops and grains like sugarcane, corn, wheat and maize is an economical and efficient method for bioethanol production. The use of these sugars for producing bioethanol leads to opportunities for farmers by increasing demand for their products, resulting in a boost in rural economies (Olfert *et al*., 2007).

Starch, a macromolecular polymer of glucose units, is a significant component of domestic and commercial waste and a useful resource that can be converted into ethanol. Yeast are unable to consume raw starch and hence, the starch must first be broken down into simple sugars (Birol *et al*., 1998). This task is achieved by two enzymes: α-amylase, which hydrolyzes α-1, 6 linkages and glucoamylase, which hydrolyzes α-1, 4 linkages in starch molecules. These enzymes are expensive and contribute significantly to ethanol production costs.

Improvement in ethanol production by using genetically engineered yeast cells in fermentation processes may lead to a boost in the fuel alcohol industry. One aspect of this project furthers the development of a yeast compatible plasmid to convert starch directly into

ethanol. The recombinant plasmid containing the TEF1 promoter, Bsd resistance, a secretion signal for the amylase, the barley α-amylase gene and an anchoring agglutinin gene to fix the protein to the outer surface of the yeast cell membrane, has been designed and created in Dr. Roesler's lab (Liao, B., 2008). Fermentation studies using this recombinant strain of yeast were undertaken to test the ability of the plasmid-containing cells to grow on glucose and soluble starch and produce ethanol.

Moreover, in industrial processes, starch is first cooked at high temperatures to solubilize it, followed by high temperature enzymatic liquefaction (α-amylase) and saccharification (glucoamylase). This process has a high energy demand and requires special equipment like heat exchangers and steam jet cookers (Williams, J., 2006). Compared to soluble starch, the use of starch particles for bioethanol production by fermentation is more economical because it avoids this cooking process and therefore, saves time and energy. This method of raw starch hydrolysis is referred to as cold hydrolysis.

A significant aspect in the fermentation of biomass to ethanol is the cost of the medium used. In previous research converting starch particles to bioethanol, expensive nutrients (yeast extract and commercial α-amylase) were used. Developing a cost-effective fermentation medium for starch particles with high efficiency of ethanol production still remains an outstanding challenge.

In this work, batch fermentation runs were performed to produce bioethanol using three substrates - glucose, soluble starch and starch particles. Two strains of *Saccharomyces cerevisiae*, NRRL Y132 and Muntons Active Brewing Yeast were used in this study. A recombinant NRRL Y132 strain, designed and developed in Dr. Roesler's lab, was used to perform batch runs on glucose and on soluble starch. A novel, inexpensive medium was formulated and batch runs were performed using this new medium. Simultaneous hydrolysis and fermentation of starch particles was also studied.

1.2 Objectives

In order to reduce the cost of the bioethanol process, the objectives of this project were:

(a) Test the ability of a new plasmid-bearing strain of *S. cerevisiae* to produce ethanol.

(b) Optimize ethanol production from starch particles by developing a cost-effective fermentation medium.

2.0 Background and Literature Review

2.1 Ethanol Production by Fermentation

Fermentation is one of the oldest biochemical processes known. It is used to produce a variety of products, including foods, flavorings, beverages, pharmaceuticals, and value-added chemicals like ethanol. The future of the fermentation industry with respect to bioethanol production depends on three major strategies. First, its ability to exploit a variety of microorganisms that are capable of efficient ethanol production by fermentation; second, to utilize various substrates such as sugars, starches or celluloses derived from a variety of different sources; and third, since utilizing starches and celluloses requires enzymes, to locate, develop and investigate relatively inexpensive sources of enzymes.

2.1.1 Microorganisms

Both yeast and bacteria are capable of efficiently converting sugars to ethanol by fermentation processes. The expansion of the ethanol industry requires the search for new and more efficient ethanologenic microorganisms. This section will highlight the varieties of bacteria and yeast that can be used for ethanol production.

A large number of bacteria are capable of ethanol production. But most of them produce other end products like butanol, isopropylalcohol, acetic acid, formic acid, arabitol, glycerol, acetone, methane, etc., as well as ethanol. Bacteria that produce ethanol as the major product (i.e. a minimum of 1 mol ethanol produced per mol of glucose utilized) are shown in Table 2.1 (Kang's homepage, 2008).

Zymomonas mobilis is a bacterium that has been extensively investigated with regard to bioethanol production. It is suitable for ethanol production due to its greater tolerance to high ethanol concentrations as compared to traditional *Saccharomyces* yeast. Ruanglek *et al*. (2006) reported that in the USA and Brazil, *Zymomonas mobilis* is used for ethanol production from corn steep liquor. Tano *et al*. (2000) studied fermentation by *Zymomonas mobilis* CP4 using sugar cane juice. However, a disadvantage of this bacterium is that it is capable of fermenting only glucose, fructose and sucrose (Ruanglek *et al*., 2006).

Table 2.1: Bacterial Species Which Produce Ethanol as the Main Fermentation Product

Bacteria	mmol Ethanol Produced per mmol Glucose Metabolized
Clostridium sporogenes	up to 4.15 [a]
Clostridium indolis (pathogenic)	1.96 [a]
Clostridium sphenoides	1.8 [a]
Clostridium sordelli (pathogenic)	1.7
Zymomonas mobilis (syn. Anaerobica)	1.9 (anaerobe)
Zymomonas mobilis *Ssp. Pomaceas*	1.7
Spirochaeta aurantia	1.5
Spirochaeta stenostrepta	0.84
Spirochaeta litoralis	1.1
Erwinia amylovora	1.2
Leuconostoc mesenteroides	1.1
Streptococcus lactis	1.0
Sarcina ventriculi (syn. Zymosarcina)	1.0

(a) In the presence of high amounts of yeast extract
Source: Kang's homepage (accessed on June 10th, 2008)

Yeast is the most commonly used microorganism for ethanol production by fermentation. The most widely used and popular biological agents of wine and beer fermentations are yeasts of the genus *Saccharomyces*. As mentioned earlier in chapter 1, there are certain unique properties of this genus that make it not only capable, but outstanding for ethanol production. Some of these properties are: fast growth rates, efficient glucose repression, efficient ethanol production and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels (Piškur *et al*., 2006). Some examples of yeasts used for ethanol production by fermentation are *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, and *Saccharomyces diastaticus*. The following sections of this chapter provide examples of various yeast strains that have been used for ethanol production by fermentation. Verma *et al*. (2000) reported the use of *Saccharomyces diastaticus* for conversion of starch to ethanol, after pretreatment of starch with α-amylase. They also studied ethanol

production from a co-culture of *Saccharomyces diastaticus* and *Saccharomyces cerevisiae* 21 strains. They used raw unhydrolyzed starch and performed simultaneous saccharification and fermentation. They reported that greater amounts of ethanol were produced using the co-culture as compared to the individual microorganisms.

2.1.2 Substrates

Ethanol by fermentation processes can be produced from any and every material that contains sugars. To make ethanol production by fermentation an economically feasible process on an industrial scale, the use of inexpensive substrates and the maximization of substrate utilization and conversion are significant aspects. Lee *et al*. in 1995 reported that a variety of possible substrates have been studied for large-scale ethanol production. Some of them are corn residue prehydrolysate, sugar beet molasses, sugar cane molasses, Jerusalem artichoke juice, cellulose, barley and cassava (Lee *et al*., 1995).

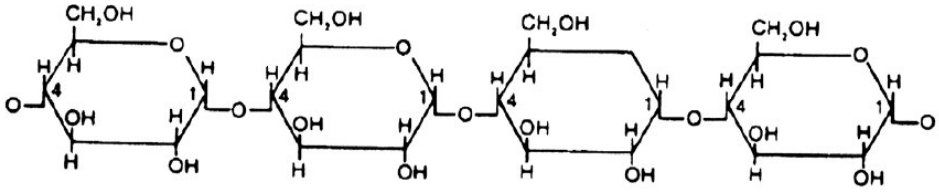
The raw materials used in ethanol production via fermentation are classified under three groups: sugars, starches, and cellulose materials. The sugars present in sugar cane, sugar beets, molasses, fruits, etc. can directly be converted to ethanol. Starches from potatoes, root crops and grains like wheat, corn, etc., need to be hydrolyzed to simple fermentable sugars by the enzymes α-amylase and glucoamylase, before they can be converted to ethanol. Similarly, cellulose from wood and other agricultural residues must be converted to simple sugars by the action of mineral acids or cellulases. Mixed substrates such as glucose-xylose mixtures, glucose-fructose mixtures, and glucose-galactose mixtures have also been investigated (Lee *et al*., 1995).

On a laboratory scale, glucose is commonly used as a substrate to study bioethanol production. A few literature examples are highlighted here. Cot *et al*. in 2007 performed aerated fed-batch fermentation on 2 % glucose with *Saccharomyces cerevisiae* strain CBS 8066. The production phase was not coupled to the growth phase and they found that 20 % (v/v) ethanol was produced in 45 hours (Cot *et al*., 2007). Hill *et al*. (1990) reported results of 14 batch runs performed on glucose at a temperature of 30°C and a pH of 4.0, using *Saccharomyces cerevisiae* strain NRRL Y132. They modelled the data and found the best value of the Monod constant to be 2 g/L.

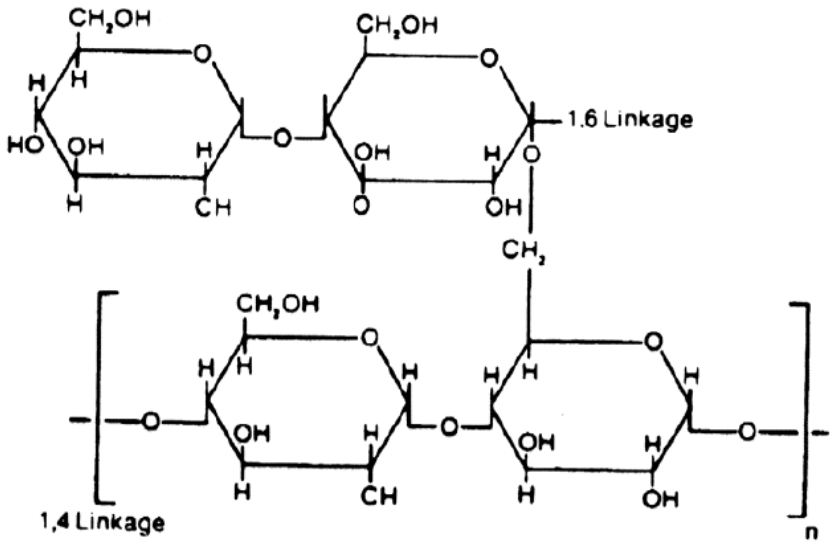
In industry, molasses, a by-product of the sugarcane industry, is the most widely used sugar for ethanol fermentation. This molasses contains approximately, by weight, 35 – 40 %

sucrose, 15 – 20 % invert sugars such as glucose and fructose and 28 – 35 % non-sugar solids (Kang's homepage, 2008). Govindaswamy *et al*. (2007) performed fermentation experiments on glucose and xylose alone as well as on combinations of both glucose and xylose. They obtained maximum specific growth rates of 0.291 h^{-1} and 0.206 h^{-1} for experiments performed on 20 g/L glucose and 20 g/L xylose, respectively. In medium containing combinations of glucose and xylose, they found that glucose was exhausted first followed by xylose.

Starch is a polymer of glucose units and it is a carbohydrate produced by most plants. There are two forms of starch, namely, amylose and amylopectin. Amylose is a straight chain form containing only α-1,4 linkages, while amylopectin is a branched chain form containing both α-1, 4 linkages and α-1, 6 linkages. The two forms are shown in Figure 2.1 (Archer Daniels Midland brochure, undated).



(a)



(b)

Figure 2.1: The two forms of starch (a) Amylose and (b) Amylopectin

The four major sources of starch are wheat, corn, tapioca and potato. Table 2.2 shows the composition of unprocessed wheat and corn (Archer Daniels Midland brochure, undated).

Table 2.2: Constituents in Unprocessed Wheat and Corn

	Wheat	**Corn**
Moisture	13 %	13 %
Starch	72 % (dry basis)	70 % (dry basis)
Protein	14 % d.b.	10 % d.b.
Fiber	7 % d.b.	10 % d.b.
Oil	2 % d.b.	5 % d.b.
Sugars	3 % d.b.	3 % d.b.
Minerals	2 % d.b.	2 % d.b.

Figure 2.2 shows a sketch of granules of the four major starch sources (Archer Daniels Midland brochure, undated). It depicts the diversity in the shape and size of starch particles.

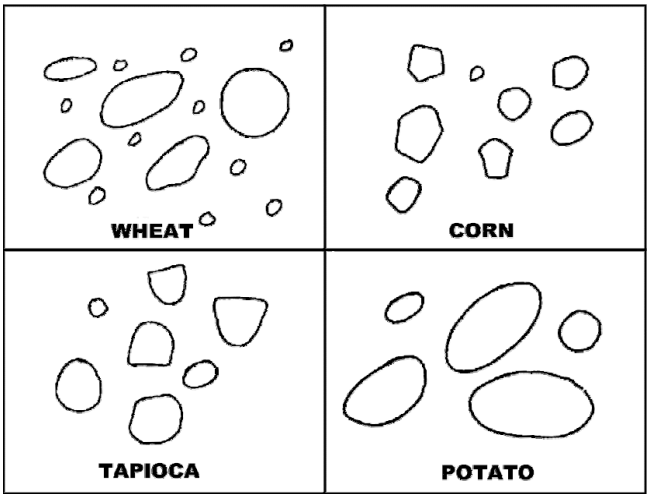


Figure 2.2: Starch Granules

Unmodified starch is insoluble in cold water. On heating the starch slurry, when a temperature of 55 – 60°C is reached, the starch granules start to swell. Figure 2.3 shows the starch granule gelatinization process (Archer Daniels Midland brochure, undated).

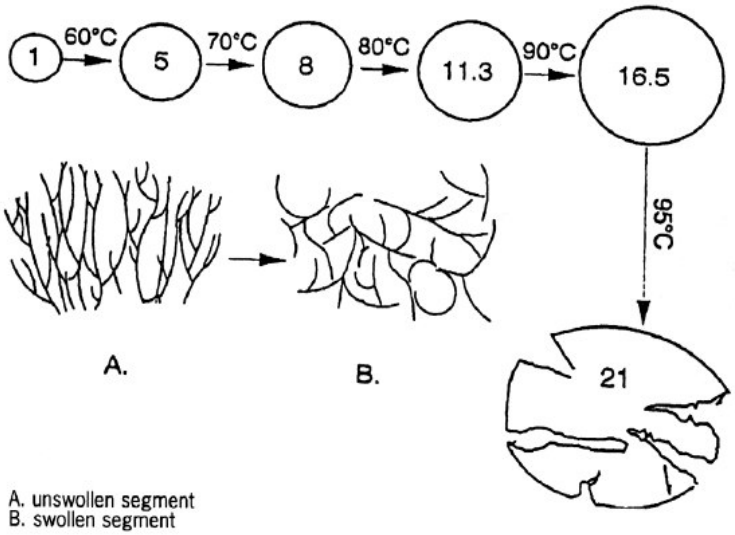


Figure 2.3: Diagrammatic Sketch of the Swelling of a Starch Granule

Lee *et al*. in 1995 studied ethanol production by fermentation using tapioca starch. They reported that liquefaction and saccharification of tapioca starch resulted in a glucose-maltose mixture containing approximately 92 % glucose and 8 % maltose. They proposed a model that accurately represents ethanol production from a mixture of glucose and maltose as substrates. Table 2.3 shows their model parameter results (Lee *et al*., 1995).

Table 2.3: Results of Model Parameter Estimation

Parameters	Estimated
μ_{Gmax}	0.401 [h^{-1}]
μ_{Mmax}	0.353 [h^{-1}]
K_G	3.0 [g/L]
K_M	9.0 [g/L]
$Y_{X/G}$	0.109
$Y_{X/M}$	0.104
$Y_{E/G}$	0.429
$Y_{E/M}$	0.451
X_{max}	183 [g/L]
E_{max}	90 [g/L]
k_1	0.5 [g/L]

There is a large amount of literature on ethanol production by fermentation where soluble starch has been used as substrate. Some of these literature examples where soluble starch has been used are discussed in section 2.2 which deals primarily with the use of genetically engineered yeast cells. Also starch particles have been used for ethanol production using the cold hydrolysis technology along with simultaneous saccharification and fermentation. Some literature examples of the use of starch particles for ethanol production are presented in section 2.4. The use of cellulose-based substrates is discussed in chapter 5, section 5.2 i.e. recommendations.

2.1.3 Enzymes

Historically, plants and animals were considered the best sources of enzymes. But recently, microbial enzymes have gained importance as their production is more economical. Two major enzymes, α-amylase and glucoamylase, have important applications in several industries like baking, brewing, detergent, medicine, textile and pharmaceutical. Today, these enzymes are mostly produced from various strains of bacteria, fungi and grains like barley. As an example, Mohamed *et al*. (2007) studied optimization of growth and glucoamylase production from *Candida famata*.

The brewing industry uses these enzymes for starch hydrolysis prior to yeast fermentation for bioethanol production. The conversion of starch to sugars like glucose requires two enzymes: α-amylase and glucoamylase. The α-amylase hydrolyses α-1,4 glycosidic linkages in starch to produce glucose, maltose and dextrins and the glucoamylase hydrolysis α-1,4 linkages as well as α-1,6 linkages to produce glucose molecules from the dextrins. The α-amylase enzyme has been shown to attack both soluble starch as well as starch particles in aqueous suspension (Hill *et al*., 1997).

Textor *et al*. (1998) studied three α-amylases, namely, bacterial, fungal and barley, to hydrolyze wheat starch particles in aqueous suspension. They determined the rates of hydrolysis at various temperatures, pH, enzyme and starch concentrations and concluded that barley α-amylase gave the best hydrolysis rate when used at pH 4.5, 45°C and at starch and enzyme concentrations of 30 g/L and 8 g/L, respectively. They found that 98 % of the starch particles were hydrolyzed in 3 hours under these conditions.

2.2 Ethanol Production from Genetically Modified *Saccharomyces cerevisiae*

Several methods for production of ethanol from starch exist. These include (a) saccharification and fermentation using a mixed culture of amylolytic and ethanol-producing microorganisms, (b) use of amylolytic enzymes from bacteria and fungi, and (c) addition of glucoamylase to the fermentation broth, which is a common practice in industry. When mixed cultures are used, the ethanol yield decreases because most of the starch is consumed for the growth of the amylolytic organism (Altıntaş *et al*., 2002). Also, the industrial use of amylolytic organisms is limited due to their low ethanol tolerance (Öner *et al*., 2005; Öner, 2006). The use of enzymes, as mentioned earlier, increases the costs of the process. Hence, an alternative strategy is the development of genetically modified microorganisms that can directly ferment starch into ethanol.

Altintas, *et al.* (2002) used the recombinant yeast strain, *Saccharomyces cerevisiae* YPB-G, having both α-amylase and glucoamylase as a bifunctional fusion protein, for the direct fermentation of starch into ethanol. Their fed-batch cultures produced 0.46 g ethanol per gram of starch. They reported that glucoamylase producing recombinant yeast strains were studied for the direct fermentation of soluble starch into ethanol and they produced 44.8 g/L ethanol from 100 g/L starch. It was also reported that an ethanol concentration of 24.9 g/L was produced from 100 g/L starch in batch cultures and 28.2 g/L ethanol from 94 g/L starch in fed-batch cultures of recombinant *Saccharomyces cerevisiae* SR93.

Massachusetts Institute of Technology scientist, Hal Alper and his colleagues recently engineered a new strain of yeast that can tolerate high levels of ethanol and glucose. This strain also produces ethanol faster than the wild-type yeast (Alper *et al*., 2006). Öner *et al.* (2005) and Öner (2006) developed a respiration-deficient recombinant *Saccharomyces cerevisiae* and examined the extent to which this mutant strain improved ethanol production from starch. They showed that the ethanol production yield with the mutant was 16% higher than the wild-type strain. Khaw *et al.* (2005) developed four types of cell-surface-engineered *Saccharomyces cerevisiae*, A, B, C and D, displaying glucoamylase. System A secreted α-amylase into the culture medium and system B had α-amylase anchored to the cell surface. Systems C and D were flocculent yeast counterparts for systems A and B, respectively. They evaluated the performance of these systems in batch fermentations and proved that system A produced the maximum ethanol from raw corn starch. The results are shown in Table 2.4:

Table 2.4: Summary of batch fermentation using different arming yeast systems

System	Hydrolysis of starch (%)	Maximum ethanol concentration ($g\ l^{-1}$)	Specific ethanol production rate ($g.g\ cell^{-1}\ h^{-1}$)
A	95	51	0.18
B	40	23	0.06
C	47	23	0.06
D	44	20	0.04

Kong *et al.* (2006) developed two *Saccharomyces cerevisiae* strains, KAM-3 and KAM-11. In KAM-3, the *FPS1* gene, which encodes a channel protein responsible for glycerol export, was deleted and in KAM-11, the *GLT1* gene, which encodes for glutamate synthase, was over-expressed. They showed that the above mentioned two mutations resulted in a 14% increase in ethanol production. A flocculent *Saccharomyces cerevisiae* strain displaying cell-surface glucoamylase was also developed by Kondo *et al*. (2002). Batch fermentation studies with these cells showed a high ethanol production rate of 0.71 $g\ h^{-1}\ L^{-1}$. Birol *et al.* (1998) investigated the production of ethanol from starch in three genetically modified *Saccharomyces cerevisiae* strains, namely, YPG/AB (produces *Aspergillus awamori* glucoamylase and *Bacillus subtilis* alpha amylase), YPG/MM (produces *Aspergillus awamori* glucoamylase and mouse alpha amylase) and YPB-G (secretes a bifunctional fusion protein containing *Aspergillus awamori* glucoamylase and *Bacillus subtilis* alpha amylase activities). They inferred that YPG/AB showed the most efficient conversion of starch to ethanol because of its higher α-amylase activity.

Glucose and xylose are the most abundant fermentable sugars present in cellulosic biomass. Wild-type *Saccharomyces*, in spite of being the best sugar fermenting microorganism, is unable to ferment xylose. Ho *et al*. in 1998 developed genetically engineered *Saccharomyces* that is capable of co-fermenting glucose and xylose. They designed high copy number yeast - *E. coli* shuttle plasmids. They concluded that the recombinant strain can effectively ferment xylose in the presence as well as absence of glucose (Ho *et al*., 1998).

The instability of cell cultures containing plasmid vectors is a major problem in the commercial exploitation of genetic engineering techniques. Plasmid stability depends on the nature of the host cell, the type and size of plasmid and environmental conditions. Plasmid encoded properties may be of significant advantage to the host cell but they result in loss of

energy due to replication and expression. The robustness of cells and how the cells respond to stresses can influence the degree of instability. Thus, the competence and ability of recombinant cells to survive and grow in altered environments forms the basis for stability enhancement in fermentation systems (McLoughlin, 1994).

During growth of plasmid bearing cells, there is a significant risk of losing the recombinant plasmids from cells due to defective segregation of plasmid during cell division or structural instability of plasmid due to mutations. Also, cells with low copy number have greater probability of producing daughter cells lacking plasmids. Baheri *et al.* (2001) modeled plasmid instability in batch and continuous fermentors and predicted that both accelerating and decelerating rates of plasmid loss occur. Moreover, medium formulation has significant effect on plasmid stability. For instance, Altintas *et al.* (2002) used YE-Salts medium to support the plasmid-containing cells during fermentation and found that only 15% of the recombinant cells lost their plasmid content by the end of the 120 hour fermentation.

2.3 Fermentation Media

The key to the development of a cost-effective fermentation process is the formulation of the culture medium. This requires the use of easily available alternative nutrients that meet the microbial requirements. Ruanglek *et al.* (2006) reported that the most significant factor for improving growth rates and ethanol productivities in synthetic media is the complex nitrogen supplement. The most commonly used organic nitrogen supplement in growth medium, yeast extract, contributes to almost 50% of the overall medium cost, and is hence expensive for use in commercial bioethanol production. The authors also evaluated three different agricultural wastes as potential yeast extract replacements: a solution from a glutamate-synthesizing process; an autolysate of brewer's yeast; and a hydrolysate of fish soluble waste. They found that the use of fish soluble waste for bioethanol production using the microorganism, *Zymomonas mobilis* gave both specific growth rate and ethanol productivity comparable to that of yeast extract, thereby making it a more economical alternative than yeast extract. Moreover, they evaluated the effects of addition of various concentrations of calcium pantothenate and concluded that the addition of calcium pantothenate did not have any significant effects on growth or ethanol production. They also studied the effect of replacing ammonium sulfate with urea as it is a less expensive source of ammonium.

Another agro-industrial waste that is widely used for bioethanol production in the USA and Brazil is corn steep liquor, a by-product of the corn wet milling process. Kadam *et al.* (1997) used a low-cost medium containing 0.3% corn steep liquor and 2.5 mM $MgSO_4.7H_2O$ for simultaneous saccharification and fermentation using *Saccharomyces cerevisiae* D_5A. They reported that $MgSO_4$ in the culture medium helps the growth of *Saccharomyces* and that it is the magnesium in the yeast extract that makes it so effective. The authors concluded that the performance of this low-cost medium was similar to that of a nutrient-rich medium.

York *et al*. (2005) developed a soy-based nutritional medium for ethanol production from *E. coli* K011. The medium consisted of mineral salts, vitamins and crude enzymatic hydrolysate of soy. Fermentation times required with this soy medium were longer as compared to LB medium, but ethanol concentrations were comparable. They reported ethanol concentrations of 44-45 g/L from 100 g/L glucose. Ishizaki and Tripetchkul (1995) found that sago starch hydrolysate as the carbon source and natural rubber serum powder as a yeast extract replacement, together form an inexpensive medium for ethanol production using the bacterium *Zymomonas mobilis*. They concluded that using this new medium gave similar glucose uptake rates, ethanol production rates and ethanol yields as compared to the values obtained on pure glucose-yeast extract medium.

2.4 Fermentation of Starch Particles

As mentioned earlier, yeast cells are unable to act on starch particles to produce ethanol. The starch must first be broken down to simpler sugar chains called dextrins. These dextrins need to be broken down to simple sugars like glucose for the yeast to be able to convert this glucose to ethanol by fermentation. This breakdown of starch is called hydrolysis. In industrial processes, starch is boiled at high temperatures (> 90°C) to make it soluble and this is followed by high temperature enzymatic liquefaction (α-amylase) and saccharification (glucoamylase) (Textor *et al.*, 1998).

It was reported that there is an overall energy savings of 17% in the process of ethanol production only by lowering the starch cooking temperature (Textor *et al*., 1998). Mikuni *et al.* (1987) stated that there could be an energy savings of approximately 40% by eliminating the cooking step. Apart from the fact that the cooking process has a high energy demand, there are some other advantages of eliminating the cooking step. First, the value of co-products is

increased as proteins undergo less thermal stress. It has been reported that the value of undenatured proteins is two to three times greater than that of denatured proteins. Second, the possibility of reusing the enzymes increases as there is decreased denaturing of enzymes (Textor *et al*., 1998). Since this cooking process requires large amounts of energy, the use of starch particles, instead of soluble starch, for bioethanol production by fermentation is more economical as it eliminates this cooking process and saves time and energy. This method of raw starch hydrolysis is referred to as cold hydrolysis.

Textor *et al.* (1998) studied the cold enzyme hydrolysis of raw wheat starch granules using barley α-amylase. They found that 98% of the starch granules were hydrolysed in 3 hours and hence concluded that barley α-amylase was the most efficient at pH 4.5 and 45°C and starch and enzyme concentrations of 30 and 8 g/L, respectively. Lang *et al.* (2001) studied hydrolysis of raw wheat starch granules and showed that in a batch system, 100 g/L of starch particles produce 80 g/L sugars in 30 hours at 45°C. Wheat starch particles are hydrolyzed by barley α-amylase below 52°C, which is the gelatinization temperature. It has also been reported that the rate of starch particle hydrolysis increases with temperature but as the temperature is increased, enzyme degradation also increases (Hill *et al*., 1997).

Lang *et al.* (2001) used a recycle bioreactor for bioethanol production from raw wheat starch particles. They found that 95% of the starch particles were converted to ethanol with 24 hours and the ethanol yield was 0.48g ethanol / g glucose. With a view to test the long term stability of the simultaneous hydrolysis and fermentation process, they performed a series of sequential batch hydrolysis and fermentation runs and obtained an overall ethanol yield of 0.49 g ethanol / g glucose. They used 3.0 L of medium containing 130 g/L starch particles, 30 g/L barley α-amylase and 1 g/L glucoamylase. They were able to recycle the enzymes and perform four sequential operations within 110 hours. Figure 2.4 presents a graph of their results.

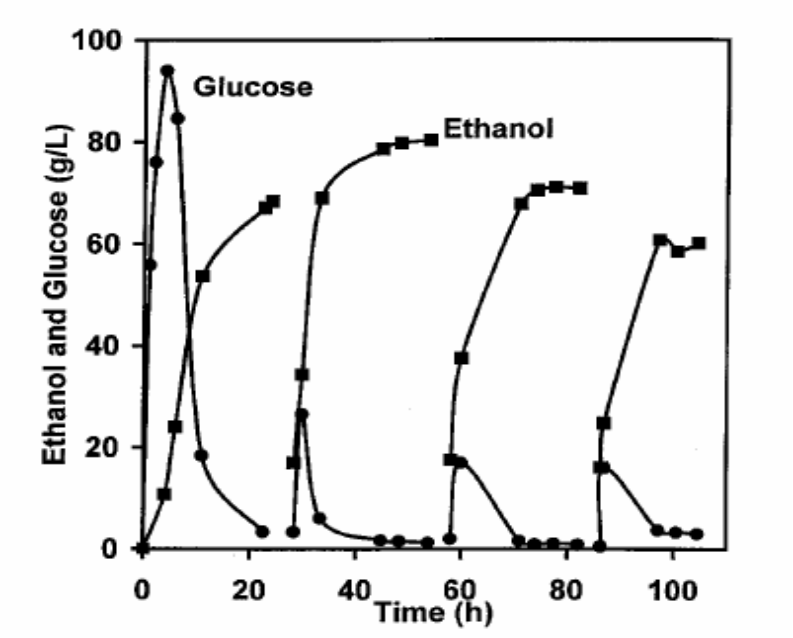


Figure 2.4: Sequential, simultaneous hydrolysis and fermentation experiments using Muntons yeast and 130 g/L starch particles, to produce 70 g/L ethanol (Lang *et al*., 2001)

Other sources of starch have also been used for bioethanol production by fermentation. Starch granules from cassava, corn, babassu and potato have been used (Lang *et al.*, 2001). Ueda *et al.* (1981) used raw cassava root starch for fermentation at pH 3.5 and 30°C for 5 days to produce ethanol with yields between 82.3% and 99.6% of the theoretical value. Mikuni *et al.* (1987) performed batch runs for simultaneous saccharification and fermentation of corn starch granules using *Saccharomyces cerevisiae*, at pH 5.0 and 30°C and achieved ethanol yields between 63.5% and 86.8% of the theoretical value. Lang *et al*. (2001) also reported that a two phase aqueous system containing polyethylene glycol enzymatically converted starch particles to glucose. Batch experiments using α-amylase immobilized on hollow fibers have also been performed. They achieved complete starch particle hydrolysis in 4 hours.

3.0 Materials and Methods

This chapter deals with the microorganisms, substrates, enzymes, media and other chemicals required while performing the fermentation experiments. The chemicals were purchased from VWR, unless otherwise mentioned. It also describes the experimental procedures performed. Calibration curves for ethanol, glucose and starch measurements are also presented in this section. In this thesis, 'biomass concentration' refers to the yeast cell concentration.

3.1 Microorganisms and Fermentation Media

3.1.1 Microorganisms:

With glucose as substrate, runs were performed using commercially available Muntons Active Brewing Yeast (Ireland), wild-type yeast strain NRRL Y132 (U.S. Department of Agriculture, Peoria, IL) and the recombinant NRRL Y132 strain (Dr. W. Roesler, University of Saskatchewan). Soluble starch fermentation runs were performed using the wild-type NRRL Y132 strain as control and the recombinant NRRL Y132 strain. The Muntons Active Brewing Yeast was used for all the starch particle fermentation runs.

3.1.2 Fermentation Media:

The two types of media used in this study have been classified as 'expensive' and 'inexpensive'. Their compositions are as follows:

The 'expensive' medium was similar to that of Thatipamala *et al.* (1992). A 100 g/L glucose medium solution contained (per liter of distilled water): 100 g of glucose; 10 g of yeast extract (technical grade, Difco laboratories); 2.5 g of ammonium chloride; 2.91 g of disodium hydrogen phosphate; 3.0 g of potassium dihydrogen phosphate; 0.25 g of magnesium sulfate; 0.08 g of calcium chloride; 4.3 g of citric acid and 3.0 g of sodium citrate. For the 20 g/L glucose medium solution, 3.0 g/L of yeast extract was used, while other nutrient concentrations were left unchanged. The amounts of nutrients (excluding the buffer components) were increased proportionately when preparing 200 g/L glucose medium solution. Soluble starch runs were performed using medium containing 20 g/L soluble starch and 3.0 g/L of yeast extract. The composition of the medium was same as that mentioned above. Soluble starch solution was prepared by mixing the correct amount of starch particles (dried overnight in a vacuum oven at

65°C) in distilled water and the solution boiled on a hot plate for 30 minutes. To account for loss of water due to evaporation, distilled water was added to bring the solution up to the correct volume. Soluble starch runs were performed using only 20 g/L soluble starch because higher concentrations of soluble starch solutions are difficult to prepare and handle due to viscosity problems. For the starch particle runs performed using the expensive medium, the medium was similar to that of Thatipamala *et al.* (1992) as mentioned above.

A novel, 'inexpensive' medium was formulated which contains cheaper ingredients as compared to the expensive medium. A detailed description of the chemical composition of the inexpensive medium is discussed in Appendix B. A 100 g/L starch particle inexpensive medium solution contained (per liter of distilled water): 100 g of starch particles (dried overnight in a vacuum oven at 65°C); 10 g of soluble garden fertilizer (Plant-Prod® Fertilizer), 0.16 g of calcium chloride and 1.25 g of Fermaid K (Lallemand, Montreal, QC). In the inexpensive medium formulation, the proportions of soluble fertilizer and Fermaid K used were based on the substrate concentration. For the 20 g/L starch particle run, 0.25 g/L of Fermaid K and 2 g/L of soluble fertilizer were used and for the 200 g/L starch particle run, 2.5 g/L of Fermaid K and 20 g/L of soluble fertilizer were used. For glucose runs performed in the inexpensive medium, the composition was the same, except that the 100 g/L starch particles were substituted by 100 g/L glucose (also dried overnight in a vacuum oven at 65°C).

3.2 Experimental Setup and Parameters

3.2.1 Bioreactor

A New Brunswick Scientific Bioflo III bioreactor was used in this study. Figure 3.1 shows a picture of the bioreactor. The working volume in the bioreactor was 2.0 L and 100-150 mL of yeast inoculum was added for each run.

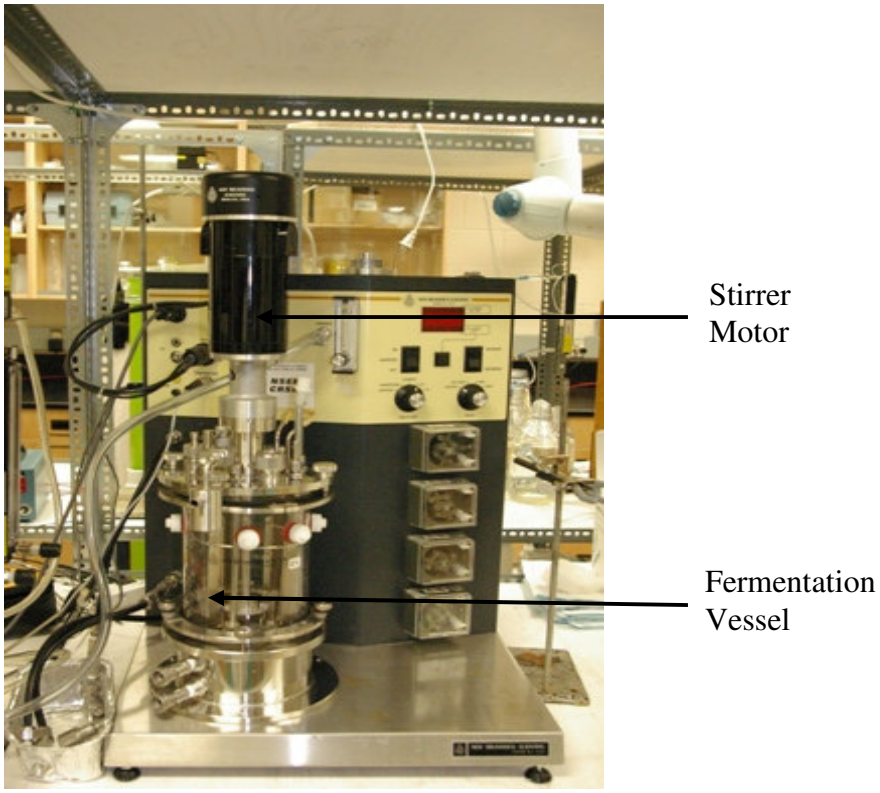


Figure 3.1: New Brunswick Scientific Bioflo III bioreactor

3.2.2 Substrates

Three different substrates were used in this study: glucose, soluble wheat starch and wheat starch particles. The glucose was purchased from VWR and the wheat starch from Archer Daniels Midland (ADM, Montreal, QC). Batch runs using glucose and starch particles were performed at three substrate concentrations, 20, 100 and 200 g/L. All batch runs using soluble starch were performed at substrate concentrations of 20 g/L.

3.2.3 Enzymes

For the glucose runs, no enzymes were required. For the soluble starch runs, α-amylase and glucoamylase enzymes were used to breakdown starch and were purchased from Sigma-Aldrich. The concentrations of α-amylase and glucoamylase were 15 g/L and 1 g/L, respectively. For the starch particle runs, the α-amylase and glucoamylase enzymes were purchased from Valley Research (South Bend, IN) and the barley malt was purchased from InfraReady Products Limited (Saskatoon, SK). The expensive medium contained Valley

Research α-amylase and glucoamylase. The novel, inexpensive medium contained ground barley malt and glucoamylase. The expensive medium was used for batch runs at different substrate concentrations and the results were compared with those of the same substrate concentrations using the novel, inexpensive medium. For the 20 g/L and 100 g/L starch particle runs, 30 g/L of α-amylase for the expensive medium runs and 30 g/L barley malt for the inexpensive medium runs was used. One g/L of glucoamylase was also added for the 20 g/L and 100 g/L runs. For the 200 g/L starch particle runs, the initial enzyme concentrations were doubled.

3.2.4 Temperature, pH and Agitation Rates

For all of the glucose and soluble starch runs, the bioreactor was operated at a temperature of 30°C and agitation of 375 RPM. The air flow rate was set to 0.02 L/minute. The starch particle batch runs were also performed at the same conditions as mentioned above. But for the sequential hydrolysis and fermentation runs using starch particles, the temperature was varied to improve productivity. During the initial hydrolysis phase, 3 hours prior to inoculation, the bioreactor was operated at four different temperatures, 30°C, 37.5°C, 45°C and 52.5°C. However, all fermentations were performed at 30°C and the agitation rate was always kept at 375 RPM.

3.3 Experimental Procedures

Before starting the batch fermentation runs, the apparatus was checked for ethanol stripping. The bioreactor was filled with 1.8 L water and 0.2 L of 100 % ethanol. A sample was collected from the bioreactor at time zero and a second sample was collected from the bioreactor after approximately 24 hours. The samples were analyzed for ethanol concentration using a gas chromatograph and it was observed that both samples had almost the same ethanol concentrations (only 1.2 % difference). Hence, it was concluded that at 30°C and at an air flow rate of 0.02 L/min, no ethanol stripping occurred.

Approximately 18-20 hours before each batch run was to be started, a yeast inoculum was started. An Erlenmeyer flask containing 100 mL of yeast medium was taken and 2.0 g of dried glucose was added to it. Then a centrifuge tube containing 1.5 mL of concentrated yeast

solution was taken from the freezer and thawed. This 1.5 mL concentrated yeast solution was added to the Erlenmeyer flask. This entire procedure for starting the yeast inoculum was performed in the biosafety cabinet. The Erlenmeyer flask was placed on a shaker at room temperature and the next day the inoculum was ready to be used for a batch run.

Also, on the day before each run, 2.0 L of fresh medium was prepared and autoclaved for 30 minutes at 121°C. The same procedure was followed in case of the novel, inexpensive medium, except that the Fermaid K was added after autoclaving and after the medium had cooled down to room temperature, as the Fermaid K may be deactivated by the high temperature of the sterilization procedure. After the addition of Fermaid K to the medium before each run, 0.025 M hydrochloric acid was used to adjust the pH of the medium to 4.0. On the day before each run, glucose or starch, depending on what substrate was to be used for the next run, were kept in the vacuum oven at 65°C to dry overnight.

3.3.1 Batch Runs with Glucose as Substrate

Batch fermentation runs using three different glucose concentrations of 20 g/L, 100 g/L and 200 g/L were performed. The required amount of glucose (for instance, 40.0 g in 2.0 L medium for the 20 g/L run) was weighed and added to the 2.0 L medium in the biosafety cabinet. This solution was vigorously stirred to make sure all of the glucose was dissolved and then poured into the bioreactor. The bioreactor was sealed and turned on. The temperature was set to 30°C, the agitation rate was set to 375 RPM and the air flow was turned on and set to 0.02 L/minute. The temperature was controlled by circulation of tap water in and out of the fermentor. Once the temperature reached 30°C, a sample was collected from the bioreactor as 'control' to measure initial glucose concentrations. Next, 100 mL of yeast inoculum was poured into the bioreactor and was allowed to mix and spread evenly into the medium. After 5 minutes, a 3 mL sample was collected from the bioreactor and this was called the 'zero' time sample. Thereafter, 3 mL samples were collected at various time intervals as the yeast cells multiplied. The optical density was measured immediately for every collected sample and the remaining samples were filtered using 0.2 μm cellulose nitrate membrane filters (Whatman) and stored in the freezer for ethanol and glucose analyses.

3.3.2 Batch Runs with Soluble Starch as Substrate

Five batch runs were performed using expensive medium and 20 g/L soluble starch as substrate. The first run was performed with the wild-type NRRL Y132 cells using only α-amylase and the second run was performed using only glucoamylase. The third run was performed using wild-type NRRL Y132 cells with both α-amylase and glucoamylase, followed by the fourth run using recombinant NRRL Y132 cells with glucoamylase alone. The fifth run was performed using recombinant NRRL Y132 cells with both α-amylase and glucoamylase.

Soluble starch solution was prepared by mixing 40 g of starch particles (dried overnight in a vacuum oven at 65°C) in 1.0 L distilled water and the solution boiled on a hot plate for 30 minutes. To account for loss of water due to evaporation, distilled water was added to bring the solution up to the correct volume i.e. 1.0 L. In the biosafety cabinet, this solution was mixed with 1.0 L of autoclaved yeast medium to finally get 2.0 L of 20 g/L soluble starch medium. This 1.0 L yeast medium contained all of the expensive medium ingredients proportionate for 2.0 L yeast medium, but the amount of distilled water was 1.0 L instead of 2.0 L, since this solution was to be mixed with the soluble starch solution.

The 2.0 L of 20 g/L soluble starch medium were poured into the bioreactor. The bioreactor was sealed and turned on. The temperature was set to 30°C, the agitation rate was set to 375 RPM and the air flow was turned on and set to 0.02 L/minute. The cold water tap was turned on for circulation of water in and out of the fermentor. Once the temperature reached 30°C, a sample was collected from the bioreactor as 'control' to measure initial starch concentrations. Then 30 g of α-amylase (i.e. 15 g/L) and 2 g of glucoamylase (i.e. 1 g/L) were added to the bioreactor. After 10 minutes, when the enzymes were well mixed into the medium, 100 mL of yeast inoculum was poured into the bioreactor and was allowed to mix and spread evenly into the medium. After 5 minutes, a 3 mL sample was collected from the bioreactor and this was called the 'zero' time sample. Thereafter, 3 mL samples were collected at various time intervals as the yeast cells multiplied. The optical density was measured immediately for every collected sample and the remaining samples were filtered using 0.2 μm cellulose nitrate membrane filters (Whatman) and stored in the freezer for ethanol analysis. At the end of the fermentation, a sample was collected from the bioreactor to be used to measure the final starch concentration.

3.3.3 Batch Hydrolysis and Fermentation of Starch Particles

Batch fermentation runs using three different starch particle concentrations of 20 g/L, 100 g/L and 200 g/L were performed using expensive as well as inexpensive medium. The temperature was also varied to improve productivity. In case of the inexpensive medium runs, during the initial hydrolysis phase, 3 hours prior to inoculation, the bioreactor was operated at four different temperatures, 30°C, 37.5°C, 45°C and 52.5°C. The actual fermentations were carried out at 30°C.

The required amount of starch particles (for instance, 40.0 g in 2.0 L medium for the 20 g/L run) were weighed and added to the 2.0 L medium in the biosafety cabinet. This solution was vigorously stirred. The 2.0 L medium containing the starch particles was poured into the bioreactor. The bioreactor was sealed and turned on. The cold water tap was turned on for circulation of water in and out of the fermentor.

For the expensive medium runs, the temperature was set to 30°C, the agitation rate was set to 375 RPM and the air flow was turned on and set to 0.02 L/minute. Once the temperature reached 30°C, a sample was collected from the bioreactor as 'control' to measure initial starch concentrations. Then 60 g of α-amylase (i.e. 30 g/L) and 2 g of glucoamylase (i.e. 1 g/L) were added to the bioreactor for the 20 and 100 g/L starch particle runs. For the 200 g/L starch particle run, 120 g of α-amylase (i.e. 60 g/L) and 4 g of glucoamylase (i.e. 2 g/L) were added to the bioreactor. After 10 minutes, when the enzymes were well mixed into the medium, 100 mL of yeast inoculum was poured into the bioreactor and was allowed to mix and spread evenly into the medium. After 5 minutes, a 3 mL sample was collected from the bioreactor and this was called the 'zero' time sample. Thereafter, 3 mL samples were collected at various time intervals as the yeast cells multiplied. The optical density was measured immediately for every collected sample and the remaining samples were filtered using 0.2 μm cellulose nitrate membrane filters (Whatman) and stored in the freezer for ethanol analysis. At the end of the fermentation, a sample was collected from the bioreactor to be used to measure the final starch concentration. A 1 mL sample was collected from the fermentor and diluted using 10 mL medium and 50 μL of this diluted sample was used for estimating cell counts using a Petrov-Hauser counting grid. Unlike the glucose and soluble starch runs, for the starch particle runs, biomass concentrations were estimated by counting cell numbers instead of using optical density measurements and dry

weight curves. This was because it is difficult to estimate biomass by measuring optical density for media containing particles.

For the inexpensive medium runs, a sample was collected from the bioreactor as 'control' to estimate initial total dry solids' concentrations. A 3 mL sample was collected, vacuum filtered and the solids collected on the 0.45 μm cellulosic filter paper were dried in a vacuum oven overnight at 65°C. The temperature of the bioreactor was set to the hydrolysis temperature i.e. 30°C, 37.5°C, 45°C or 52.5°C and 60 g of barley malt (i.e. 30 g/L) was added to the bioreactor for the 20 and 100 g/L starch particle runs. For the 200 g/L starch particle runs, 120 g of barley malt (i.e. 60 g/L) was added. The hydrolysis was carried out for 3 hours. The agitation rate was set to 375 RPM and the air flow was turned on and set to 0.02 L/minute. After the 3 hour hydrolysis, the temperature of the bioreactor was set to 30°C. Once the temperature reduced to 30°C, 2 g of glucoamylase (i.e. 1 g/L) was added to the bioreactor for the 20 and 100 g/L starch particle runs. For the 200 g/L starch particle run, 4 g of glucoamylase (i.e. 2 g/L) was added at this point. After 5 minutes, when the glucoamylase is well mixed into the fermentation broth, a 3 mL sample was collected to estimate the total dry solids' concentration. Next, 100 mL of yeast inoculum was poured into the bioreactor and was allowed to mix and spread evenly into the medium. After 5 minutes, a 3 mL sample was collected from the bioreactor and this was called the 'zero' time sample. Thereafter, 3 mL samples were collected at various time intervals as the yeast cells multiplied. The optical density was measured immediately for every collected sample and the remaining samples were filtered using 0.2 μm cellulose nitrate membrane filters (Whatman) and stored in the freezer for ethanol analysis. A 1 mL sample was collected from the fermentor and diluted using 10 mL medium and 50 μL of this diluted sample was used for estimating cell counts using a Petrov-Hauser counting grid. Also, to estimate total dry solids' concentrations, a 3 mL sample was collected from the bioreactor, vacuum filtered and the solids collected on the 0.45 μm cellulosic filter paper were dried in a vacuum oven overnight at 65°C.

3.3.4 Enzyme Activity Determinations for α-amylase

In order to determine the enzyme activity of α-amylases from various suppliers, an initial activity test was performed. A starch calibration curve was prepared at an optical density of 580 nm, which is presented in section 3.4.5.4. These activities were determined using the starch-iodine method, with initial enzyme concentrations of 0.1 mg/mL and initial soluble starch

concentrations of 0.5 g/L. The experiments were carried out in a 50°C water bath (Xiao *et al.*, 2006). This starch-iodine method is described in section 3.4.5.4.

3.4 Analysis

3.4.1 Biomass Concentrations

For the glucose fermentation runs, biomass was measured by measurement of optical densities. The optical densities were measured using a UV-VIS mini 1240 spectrophotometer (Shimadzu) at 620 nm wavelength. A graph of optical density verses biomass concentration is presented in section 3.4.5.1. Section 3.4.5.1 also describes the procedure for performing the dry weight experiments. For the soluble starch runs, the biomass was estimated in the same way using dry weight curves presented in section 3.4.5.1. For the starch particle runs, biomass was estimated using cell counts that were measured using the Petrov-Hauser counting grid.

3.4.2 Ethanol Concentrations

Ethanol concentrations were measured using gas chromatography using a capillary column with a flame ionization detector (FID). The calibration curve and the details of the method are presented in section 3.4.5.2.

3.4.3 Glucose Concentrations

Glucose concentrations were measured using a glucose analyzer, GM8 Analyser (Analox Instruments) based on the glucose oxidase principle. The procedure is presented in section 3.4.5.3.

3.4.4 Starch Concentrations

For the starch particle runs performed using the expensive medium, the initial and final starch concentrations were measured using the starch-iodine method, which is described in section 3.4.5.4 (Xiao *et al.*, 2006). For runs performed using the inexpensive medium, exact final starch concentrations could not be measured, but instead, total dry solid measurements were taken to estimate the amount of particles consumed during each run. For the soluble starch runs, the initial and final starch concentrations were not measured as an appropriate method to perform starch analysis was not yet established in the lab.

3.4.5 Calibrations

This section presents the calibration results for determination of biomass, ethanol, glucose and starch concentrations. The calibration curves presented were used to estimate all of the experimental data.

3.4.5.1 Biomass Calibration Curves

To perform dry weight experiments, the first step was to grow yeast cells in medium. Three to four 250 mL Erlenmeyer flasks were used and approximately 100 mL yeast medium containing 20 g/L glucose was poured into each of them. Three to four 2.0 mL centrifuge tubes containing frozen yeast cultures were taken from the freezer and thawed. Each centrifuge tube contained approximately 1.5 mL yeast solution. One centrifuge tube contents were emptied into each Erlenmeyer flask. The flasks were kept on a shaker at room temperature for approximately 20 hours. The contents of all flasks were mixed. The optical density of this yeast solution was measured at 620 nm using a plastic cuvette. The spectrophotometer was zeroed using distilled water. Some of this yeast solution was diluted to get 5-6 readings between the maximum optical density measured and an optical density of 0.02. The total volume of the remaining yeast solution was recorded and it was then centrifuged (5000 RPM for 10 minutes) in about 6-8 centrifuge tubes, with approximately 40 mL in each tube. After centrifuging, the liquid above the pellet is decanted off. Ten mL of water was added to each tube and vortexed. After the pellet dissolved in each tube, the tubes are centrifuged again at the same speed and for the same time. The liquid above the pellet in each tube was decanted off again and approximately 5 mL water was added to one of the tubes. This tube is vortexed until the pellet is resuspended. The solution from this tube is poured into the second tube and vortexed again to resuspend the second pellet. This procedure is repeated for all of the tubes. The final yeast solution is poured into a pre-weighed aluminum weigh-boat. This weigh-boat is kept in a vacuum oven at 65°C overnight. The weight of the dried biomass was measured and the dry biomass concentration was calculated by dividing the dry weight (in mg) by the total volume of yeast solution used (in mL). This concentration corresponds to the optical density of the original yeast solution.

The optical densities were measured using a UV-VIS mini 1240 spectrophotometer (Shimadzu) at 620 nm wavelength. A graph of optical density verses dry weight concentration was plotted for each of the three types of yeast cells used in this study: wild-type NRRL Y132,

recombinant NRRL Y132 and Muntons Active Brewing Yeast. Figure 3.2 shows the three calibration curves. The equations for Muntons yeast, wild-type NRRL Y132 yeast and recombinant NRRL Y132 yeast are valid between OD's of 0.0127, 0.0325 and 0.0370 and 1, respectively.

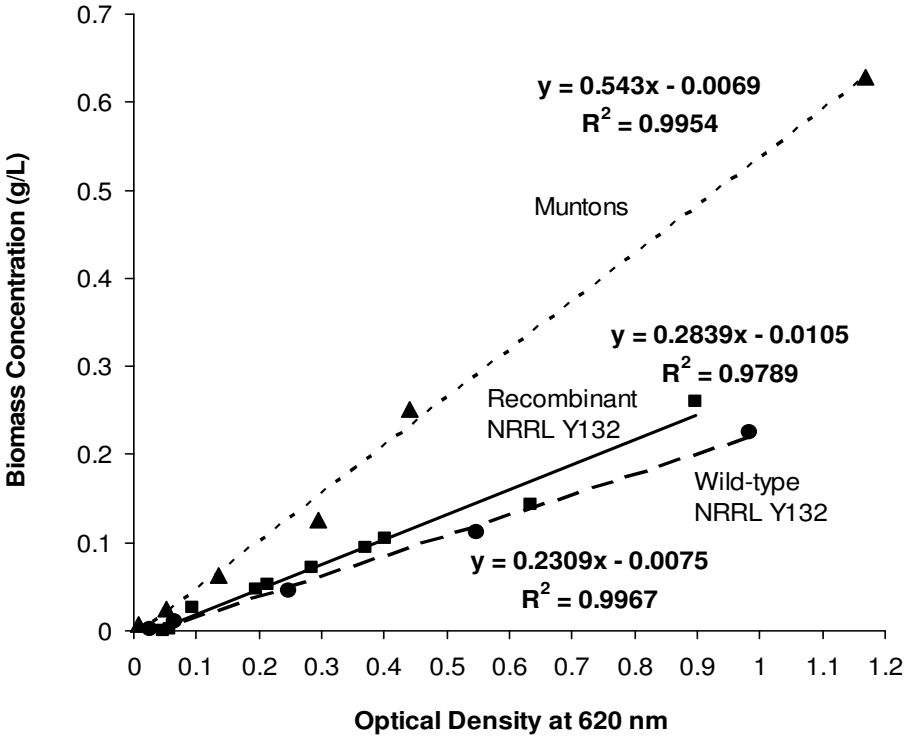


Figure 3.2: Dry Weight Concentration Curves for the three types of yeast cells

3.4.5.2 Ethanol Calibration Curve

Ethanol concentrations were measured using an Agilent 7890 gas chromatograph. A 5 % phenyl methyl siloxane column with a flame ionization detector (FID) was used. The run time was 2.15 minutes and the injection volume was 0.1 µL. The oven temperature was held at 40°C for 0.75 minutes and then increased to 110°C at the rate of 50°C /minute. The injector temperature was 250°C and the detector temperature was 300°C. The runs were performed at the 'pulsed split' mode and the split ratio was 20:1. The retention time was 0.611 minute. The calibration curve is shown in Figure 3.3. This curve is valid between an area of 124 and 5000.

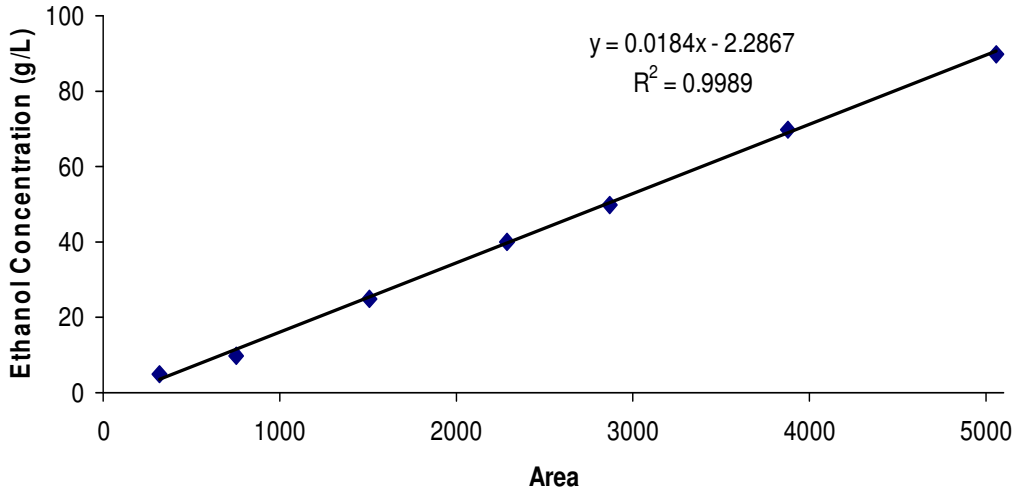


Figure 3.3: Ethanol calibration curve

3.4.5.3 Glucose Calibrations

Glucose concentrations were measured using a glucose analyzer, GM8 Analyzer (Analox Instruments) based on the glucose oxidase principle. This glucose meter is based on a 'one-point calibration' method. Using this method, 1 %, 8 % and 10 % standards were used to calibrate the machine for the 20 g/L, 100 g/L and 200 g/L runs, respectively.

3.4.5.4 Starch Calibration Curve and Measurement of Starch Concentrations in Fermentation Samples

For the starch particle runs performed using the expensive medium, the initial and final starch concentrations were measured using the iodine method (Xiao *et al.*, 2006). The procedure for the starch-iodine method is as follows:

Six mL of the initial 'control' fermentation sample was mixed with 18 mL distilled water (i.e. diluted 4 times), and boiled for 15 minutes on a hot plate with stirring. The final volume of this mixture was brought up to 24 mL using reverse osmosis water (i.e. 6 mL sample plus 18 mL water) to account for evaporation during boiling. Two mL of this solution was used for the starch analysis by the iodine method. Iodine reagent, containing 5mM iodine and 5mM potassium iodide, was prepared. Two g/L soluble wheat starch solution (to be used as a standard), 1M hydrochloric acid (HCl) and 0.1M phosphate buffer, pH 7.0, were prepared. A starch standard curve was prepared (i.e. OD_{580} nm versus starch concentration in g/L). Two mL

medium, containing no starch, was mixed with 2 mL phosphate buffer and used for zeroing the spectrophotometer.

Three samples were prepared as follows for each run:

(a) 2 mL 2 g/L starch standard solution with 2 mL phosphate buffer

(b) 2 mL of the diluted, boiled fermentation sample with 2 mL phosphate buffer

(c) 2 mL of the last sample of the fermentation run with 2 mL phosphate buffer.

All three samples were placed in a 50°C water bath for 30 minutes. One mL 1M HCl was added. This was followed by addition of 5 mL iodine reagent to each of the three samples and votexing. The absorbencies for the three samples were read at 580 nm using a spectrophotometer (samples were diluted as required to measure OD accurately and the dilution factor considered while calculating the actual starch concentration). These absorbencies were used to determine the starch concentrations using the starch standard curve.

The starch standard curve at 580 nm is shown in Figure 3.4. This curve is valid between optical densities of 0.011 and 1.

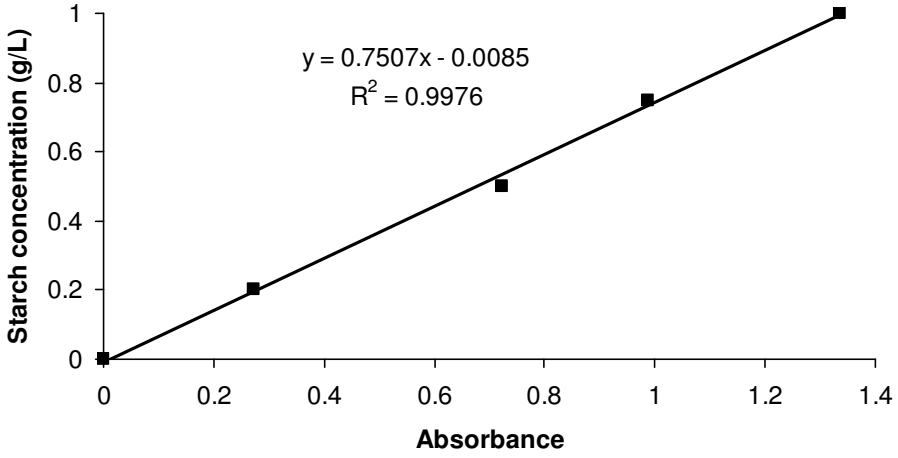


Figure 3.4: Starch calibration curve at 580 nm

4.0 Results and Discussion

Batch fermentation runs using two different media and three different substrates, namely, glucose, soluble starch and starch particles, were performed to produce bioethanol. Two strains of *Saccharomyces cerevisiae*, NRRL Y132 and Muntons Active Brewing Yeast were used in this study. Hydrolysis temperature effects on simultaneous hydrolysis and fermentation of starch particles was studied. In addition, a recombinant NRRL Y132 strain, designed and developed in Dr. Roesler's lab, was used to perform batch runs on glucose and on soluble starch. The results for all the batch runs performed using glucose as substrate, were modelled.

4.1 Reproducibility and Error

Before presenting results, it is important to present experiments and calculations performed to test the accuracy, reproducibility and error of important measurements. For the glucose runs, the error in glucose concentrations measurements was calculated. Each sample was measured three times, the mean and standard deviation for each was calculated and the percent error as well as the standard error of mean was calculated for each. The average percent error was found to be 4.6 % and the average standard error of the mean was 0.058 g/L. All of the other error calculations were performed with respect to starch particle runs with starch particle concentrations between 20 and 200 g/L. The errors in the maximum specific growth rate, the ethanol yield, the maximum ethanol concentration for each run, the total dry solids' concentration and the counting of cell numbers were calculated and the results are presented below.

The 100g/L starch particles expensive medium run was performed three times. Figures 4.1 and 4.2 show the biomass and ethanol curves, respectively, for the three runs.

From the results of all three runs, using data during the logarithmic phase, it was found that the μ_{max} was 0.094 ± 0.030 h^{-1}, the ethanol yield was 0.38 ± 0.04 g ethanol / g glucose and the maximum ethanol concentration was 46.9 ± 1.3 g/L. The errors in μ_{max}, ethanol yield and maximum ethanol concentration were ± 31.9 %, ± 10.5 % and ± 2.77 %, respectively.

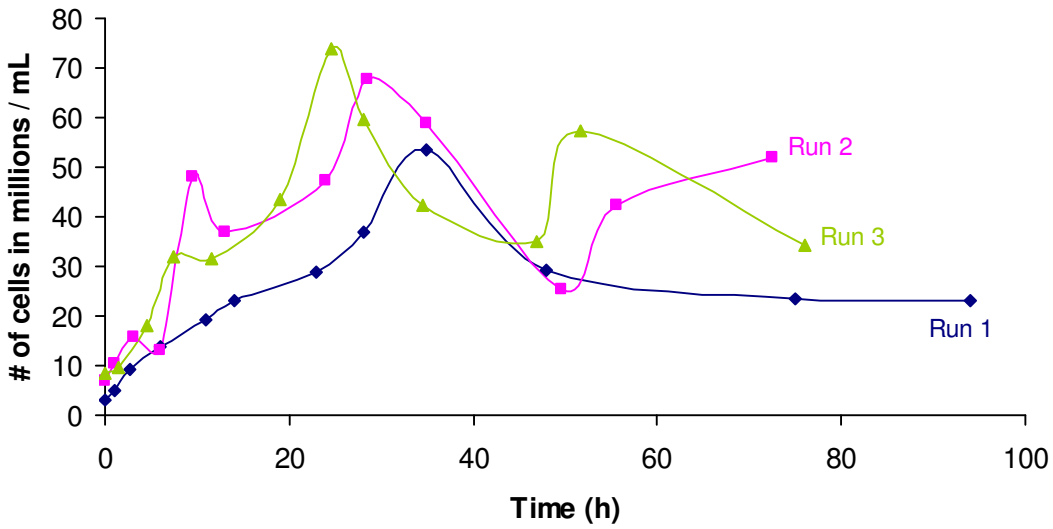


Figure 4.1: Reproducibility of biomass for identical runs using Muntons yeast in 100 g/L starch particles as substrate at 30°C

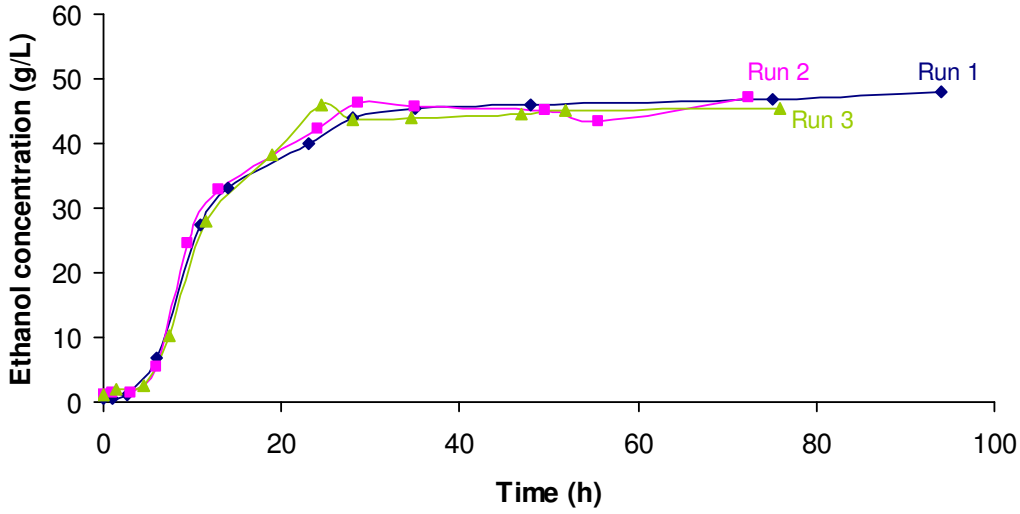


Figure 4.2: Reproducibility of ethanol for identical runs using Muntons yeast in 100 g/L starch particles as substrate at 30°C

Table 4.1 shows the initial and final starch concentrations for the three runs.

Table 4.1: Summary of Initial and Final Starch concentrations of the three 100 g/L starch particles in expensive medium runs

	Initial Starch Concentration (g/L)	Final Starch Concentration (g/L)
Run 1	123.9	5.3
Run 2	102.6	8.3
Run 3	107.6	6.2

To test the accuracy of the counting of cell numbers, the cells were counted three times for each sample. The mean cell number was plotted against time and the graph is shown in Figure 4.3.

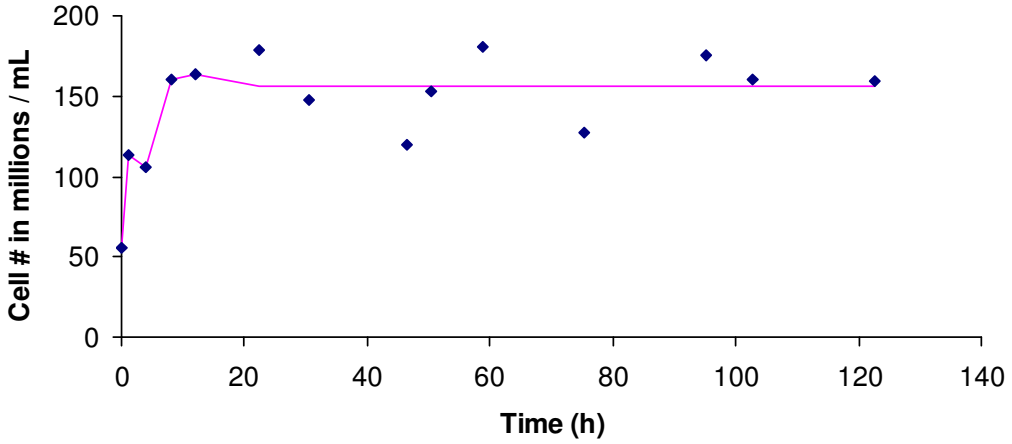


Figure 4.3: Variation of mean cell number during the 30°C run using Muntons yeast in 100 g/L starch particles as substrate, barley malt and glucoamylase

The mean (1.56×10^8 cells / mL) and the standard deviation (2.2×10^7 cells / mL) for the last nine samples were calculated and the percent error was found to be ±13.9 %. Hence, it was concluded that there was a 13.9 % error in the counting of cell numbers for biomass measurements.

To determine the error in the measurement of total dry solids, six three mL samples were collected from the fermentor. The fermentation broth contained 100 g/L starch particles and 30

g/L barley malt. Hence, the theoretical concentration of total dry solids in each of the six samples collected should be 130 g/L. The measured concentrations were 116, 119, 140, 127, 144 and 125 g/L. The mean (129 g/L) and standard deviation (11.1 g/L) of the six measurements was calculated and the percent error was found to be ± 8.6 %. The standard error of the mean was also calculated and found to be 4.5 g/L.

4.2 Comparison of Enzyme Activities of Four Sources of α-amylase

Industrial processes require α-amylase to hydrolyse starch during fermentation. It is known that α-amylase effectively acts on both soluble starch as well as starch particles in an aqueous suspension. Hill *et al*. in 1997, studied α-amylase inhibition and inactivation in barley malt during cold starch hydrolysis. They used barley α-amylase for their experiments. They concluded that this α-amylase decayed at a rate of 1.1 % h^{-1} at 45°C. They also found that the amount of glucose and maltose produced during the hydrolysis of starch particles, also affected the catalytic activity of the α-amylase. Studying phenomena that may affect the kinetics of starch hydrolysis by α-amylase is significant before designing a successful hydrolysis system (Hill *et al*., 1997).

In order to determine the enzyme activity of α-amylase from various suppliers, an initial activity test was performed. Table 4.2 shows the enzyme activities of three different α-amylases and powdered barley malt. These activities were determined using the starch-iodine method, with initial enzyme concentrations of 0.1 mg/mL and initial soluble starch concentrations of 0.5 g/L. The experiments were carried out in a 50°C water bath (Xiao *et al.*, 2006).

Table 4.2: Summary of α-amylase enzyme activities

Supplier of α-amylase	Source	ACTIVITY (g/min/g enzyme)
Crosby and Baker	Fungal	0.134
Sigma	Barley	0.096
InfraReady Barley malt	Barley	0.062
Valley Research	Fungal	0.030

Although the enzyme activity of Crosby and Baker α-amylase was the highest, this enzyme is not pure and a 5 g/L solution of it contains approximately 3 g/L glucose. Sigma no longer supplied barley α-amylase. Moreover, the most inexpensive of all four sources of α-amylase is the barley malt and hence, this was used as the α-amylase source for all of the inexpensive medium runs. For the expensive medium runs, Valley Research α-amylase was chosen. A 5 g/L solution of Valley Research α-amylase contains approximately 0.9 g/L glucose.

Since barley malt was used as the source of α-amylase for the runs using starch particles in inexpensive medium, it was decided to determine barley malt activities using various initial starch concentrations. Figure 4.4 shows the relationship between malt activity and initial starch concentration. The concentration of barley malt was 0.1 mg/mL.

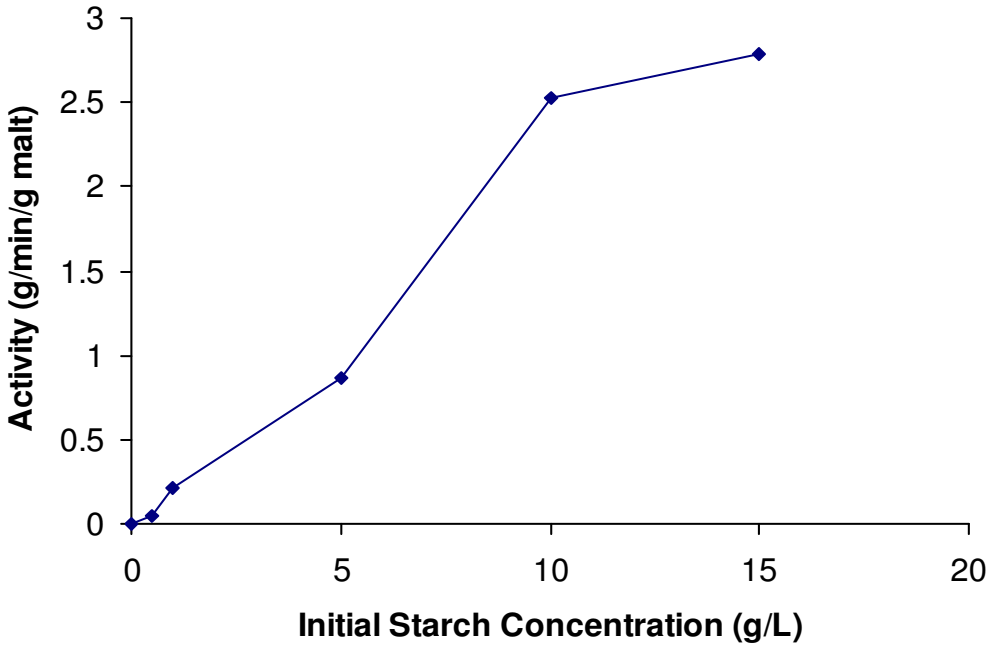


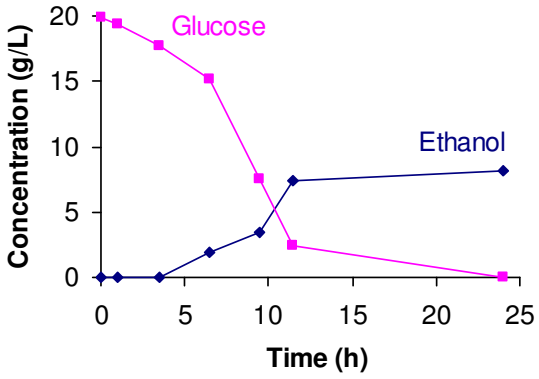
Figure 4.4: Effect of substrate (starch) on the activity of α-amylase with a barley malt concentration of 0.1 mg/mL

From the above graph it can be observed that as the initial starch concentration increases from 0 g/L to 15 g/L, the barley malt activity also increases. But from the trend, it seems that the activity may start reaching near its highest value at an initial starch concentration of approximately 10 g/L.

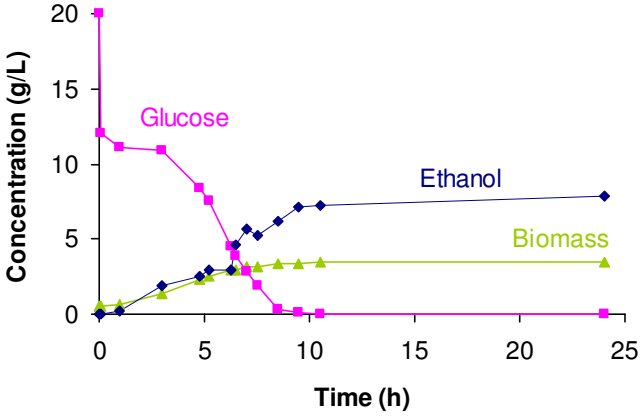
4.3 Production of Ethanol from Glucose

4.3.1 *Saccharomyces* strain NRRL Y132 vs Muntons Active brewing Yeast

Muntons active brewing yeast and wild-type NRRL Y132 yeast were cultured in the bioreactor at 30°C using expensive medium at glucose concentrations of 20 g/L, 100 g/L and 200 g/L. Figures 4.5, 4.6 and 4.7 show the biomass, glucose and ethanol concentration graphs for these batch runs.



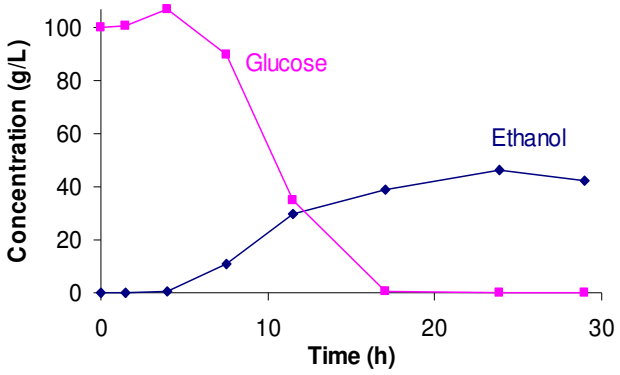
(a)



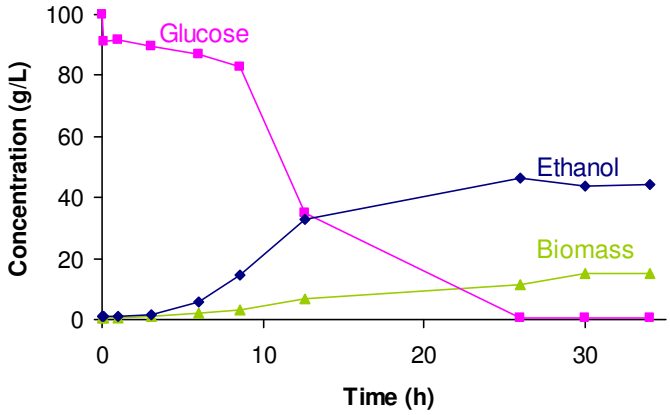
(b)

Figure 4.5: Batch growth dynamics of two yeast strains at low initial glucose concentrations of 20 g/L at 30°C. (a) Strain NRRL Y132 and (b) Muntons active brewing yeast

From Figure 4.5, it can be observed that the maximum ethanol concentrations for both the runs were similar. The maximum ethanol concentration for the 20 g/L glucose run performed with NRRL Y132 was 8.2 g/L and that performed with Muntons yeast was 7.9 g/L. So, when comparing the two strains of yeast with respect to runs performed in 20 g/L glucose medium, it was observed that the NRRL Y132 yeast produces similar ethanol as compared to Muntons yeast.



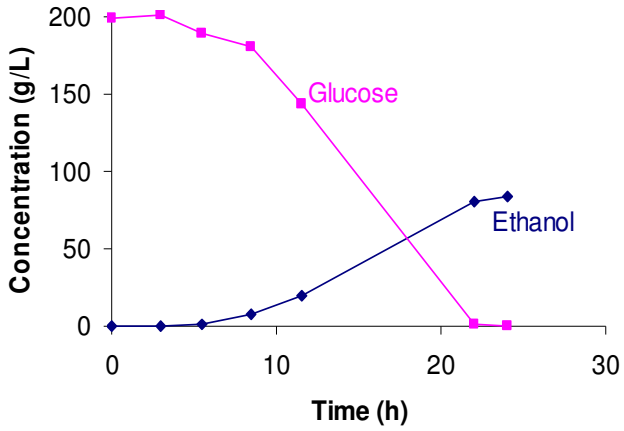
(a)



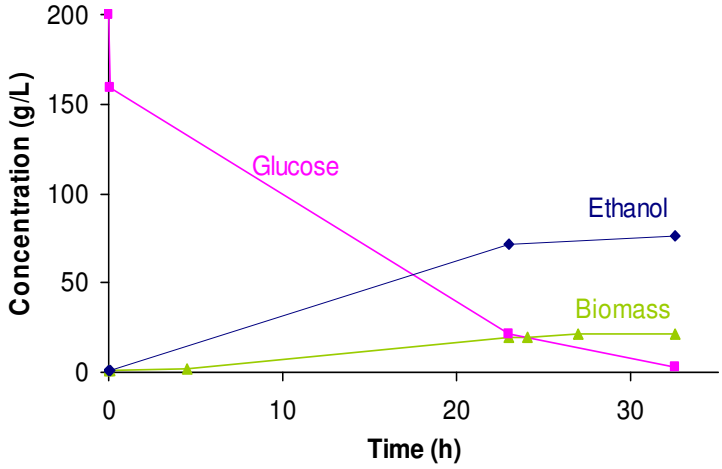
(b)

Figure 4.6: Batch growth dynamics of two yeast strains at intermediate initial glucose concentrations of 100 g/L at 30°C. (a) Strain NRRL Y132 and (b) Muntons active brewing yeast

At 100 g/L glucose, the maximum ethanol concentration for the run performed with NRRL Y132 was 46.2 g/L and that performed with Muntons yeast was 44.5 g/L. So, when comparing the two strains of yeast with respect to runs performed in 100 g/L glucose medium, it was observed that the NRRL Y132 yeast produced more ethanol as compared to Muntons yeast.



(a)



(b)

Figure 4.7: Batch growth dynamics of two yeast strains at high initial glucose concentrations of 200 g/L at 30°C. (a) Strain NRRL Y132 and (b) Muntons active brewing yeast

The maximum ethanol concentration for the 200 g/L glucose run performed with NRRL Y132 was 83.5 g/L and that performed with Muntons yeast was 76.4 g/L. Thus, when comparing the two strains of yeast with respect to runs performed in 200 g/L glucose medium, it was observed that the NRRL Y132 yeast produced more ethanol as compared to Muntons yeast. Hence, at all three glucose concentrations, the NRRL Y132 strain of *Saccharomyces cerevisiae* produced more ethanol as compared to Muntons active brewing yeast.

Table 4.3 shows a summary of the results of batch runs performed using the two different strains of *Saccharomyces cerevisiae*. The ethanol yields shown in the table are the ratios of the highest ethanol concentration produced in each batch run and the amount of glucose consumed during the run. The maximum specific growth rates were calculated using linear regression and the biomass yields were calculated by modeling of the data (shown in Appendix A).

It can be observed from the table that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the NRRL Y132 yeast were 0.42, 0.37 and 0.41 h^{-1}, respectively, while those of Muntons yeast were 0.32, 0.19 and 0.13 h^{-1}, respectively. Hence, at all three glucose concentrations, the maximum specific growth rates were significantly higher for the NRRL Y132 strain as compared to those for Muntons Active Brewing yeast. Also, at all three glucose concentrations, the ethanol yields were slightly higher for runs performed with the NRRL Y132 yeast (0.41, 0.46 and 0.42 g ethanol / g glucose) as compared to those performed with Muntons yeast (0.40, 0.45 and 0.39 g ethanol / g glucose).

Table 4.3: Summary of glucose runs with Muntons and with NRRL Y132 yeasts

	MUNTONS YEAST WITH EXPENSIVE MEDIUM	NRRL Y132 WITH EXPENSIVE MEDIUM
Glucose conc. (g/L)	20	20
μ (h^{-1})	0.32	0.42
Ethanol Yield (g ethanol/g glucose)	0.40	0.41
Final Glucose conc. (g/L)	0	0.06
Biomass Yield (g yeast/g glucose)	0.15	-
Glucose conc. (g/L)	100	100
μ (h^{-1})	0.19	0.37
Ethanol Yield (g ethanol/g glucose)	0.45	0.46
Final Glucose conc. (g/L)	0.50	0
Biomass Yield (g yeast/g glucose)	0.052	-
Glucose conc. (g/L)	200	200
μ (h^{-1})	0.13	0.41
Ethanol Yield (g ethanol/g glucose)	0.39	0.42
Final Glucose conc. (g/L)	2.6	0.10
Biomass Yield (g yeast/g glucose)	0.10	-

4.3.2 Wild-type NRRL Y132 vs Recombinant NRRL Y132

The runs performed using the wild-type NRRL Y132 strain of *Saccharomyces cerevisiae* on glucose were the necessary first steps to culturing the plasmid-containing yeast cell lines since it is necessary to know how the plasmid affects the yeast's ability to grow. The recombinant plasmid containing the barley α-amylase gene, a secretion signal for the amylase and an anchoring agglutinin gene to fix the protein to the outer surface of the yeast cell membrane, was

designed and created in Dr. Roesler's lab. This recombinant NRRL Y132 strain was designed to secrete α-amylase and fix it to the outer yeast membrane. Batch runs using this recombinant strain were performed to test the ability of the plasmid-containing cells to grow on glucose and produce ethanol. The plasmid-containing recombinant NRRL Y132 yeast was cultured in the bioreactor at the same three glucose concentrations and same medium as the wild-type.

Figures 4.8, 4.9 and 4.10 show the biomass, glucose and ethanol results for batch runs performed using the recombinant NRRL Y132 yeast at three different glucose concentrations, with the wild-type NRRL Y132 as the control.

From Figure 4.8, it can be observed that the maximum ethanol concentration for the 20 g/L glucose run performed with wild-type NRRL Y132 was 8.2 g/L and that performed with the recombinant NRRL Y132 was 10.2 g/L. So, when comparing the wild-type yeast to the recombinant one, with respect to runs performed in 20 g/L glucose medium, it was observed that the recombinant NRRL Y132 yeast produced more ethanol.

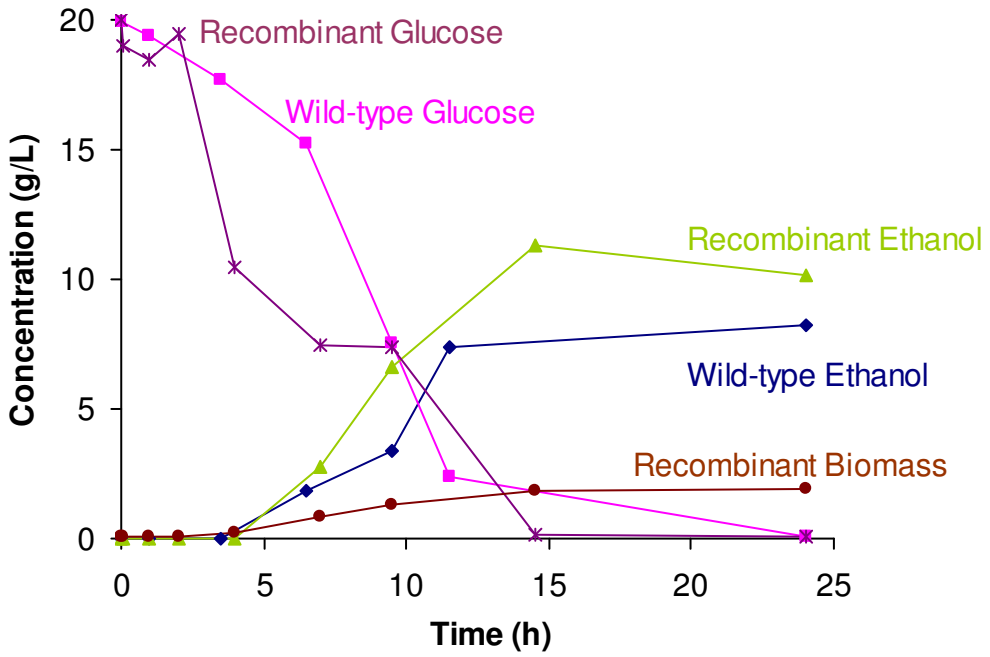


Figure 4.8: Comparison of glucose and ethanol concentration trends for the recombinant and the wild-type NRRL Y132 cells in 20 g/L glucose at 30°C

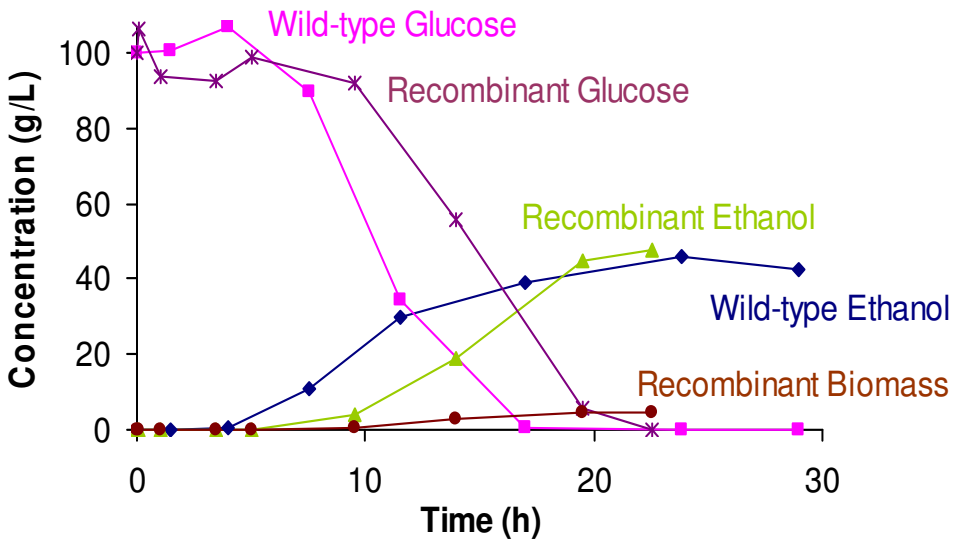


Figure 4.9: Comparison of biomass, glucose and ethanol concentration trends for the recombinant and the wild-type NRRL Y132 cells in 100 g/L glucose at 30°C

As seen from Figure 4.9, again, the maximum ethanol concentration for the 100 g/L glucose run performed with wild-type NRRL Y132 was 46.2 g/L and that performed with the recombinant NRRL Y132 was 47.8 g/L. Thus, when comparing the wild-type yeast to the recombinant one, with respect to runs performed in 100 g/L glucose medium, the recombinant NRRL Y132 yeast produced more ethanol.

From Figure 4.10, it can be observed that the maximum ethanol concentration for the 200 g/L glucose run performed with wild-type NRRL Y132 was 83.5 g/L and that performed with the recombinant NRRL Y132 was 77.6 g/L. When comparing the wild-type yeast to the recombinant one, with respect to runs performed in 200 g/L glucose medium, it was observed that unlike the 20 g/L and 100 g/L runs, in this run the wild-type NRRL Y132 yeast produced more ethanol as compared to the recombinant NRRL Y132 yeast.

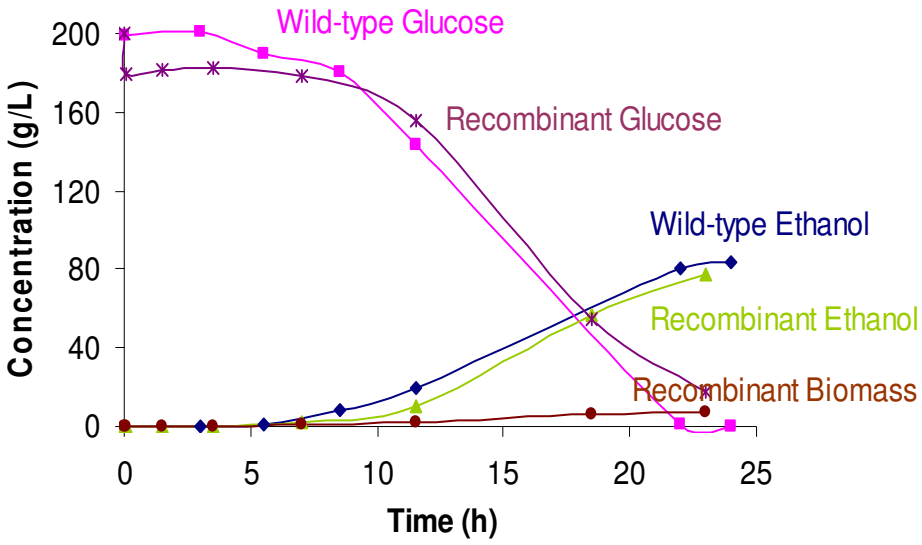


Figure 4.10: Comparison of biomass, glucose and ethanol concentration trends for the recombinant and the wild-type NRRL Y132 cells in 200 g/L glucose at 30°C

Table 4.4 shows the results of runs performed using the wild-type NRRL Y132 strain and the recombinant, α-amylase secreting, NRRL Y132 strain.

From Table 4.4, it can be observed that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the wild-type NRRL Y132 yeast were 0.42, 0.37 and 0.41 h^{-1}, respectively, while those for the recombinant NRRL Y132 yeast were 0.34, 0.33 and 0.24 h^{-1}, respectively. Hence, at all three glucose concentrations, the maximum specific growth rates were higher for the wild-type yeast as compared to those for the recombinant yeast. At glucose concentrations of 20 and 100 g/L, the ethanol yields were higher for runs performed with the recombinant yeast (0.51 and 0.48 g ethanol / g glucose) as compared to those performed with the wild-type yeast (0.41 and 0.46 g ethanol / g glucose). But at a glucose concentration of 200 g/L, the ethanol yields were the same for both the wild-type and the recombinant cells (0.42 g ethanol / g glucose). Therefore, on the whole, it can be concluded that the recombinant cells can grow and produce good ethanol yields on low, intermediate as well as high glucose concentrations.

Table 4.4: Summary of glucose runs with wild-type NRRL Y132 and with recombinant NRRL Y132 yeast

	WILD-TYPE NRRL Y132 WITH EXPENSIVE MEDIUM	RECOMBINANT NRRL Y132 WITH EXPENSIVE MEDIUM
Glucose conc. (g/L)	20	20
μ (h^{-1})	0.42	0.34
Ethanol Yield (g ethanol/g glucose)	0.41	0.51
Final Glucose conc. (g/L)	0.06	0.06
Biomass Yield (g yeast/g glucose)	-	0.093
Glucose conc. (g/L)	100	100
μ (h^{-1})	0.37	0.33
Ethanol Yield (g ethanol/g glucose)	0.46	0.48
Final Glucose conc. (g/L)	0	0.1
Biomass Yield (g yeast/g glucose)	-	0.044
Glucose conc. (g/L)	200	200
μ (h^{-1})	0.41	0.24
Ethanol Yield (g ethanol/g glucose)	0.42	0.42
Final Glucose conc. (g/L)	0.1	17.1
Biomass Yield (g yeast/g glucose)	-	0.037

4.3.3 Expensive medium vs Inexpensive medium

A novel, inexpensive medium was formulated and tested for its fermentation capacity. The preliminary glucose runs using Muntons yeast and expensive medium, discussed earlier in section 4.3.1, were compared to those performed using the novel, inexpensive medium. Muntons

yeast was cultured in the bioreactor using the inexpensive medium at glucose concentrations of 20 g/L, 100 g/L and 200 g/L.

Figures 4.11, 4.12 and 4.13 show the graphs for batch runs performed using Muntons yeast at three different glucose concentrations, in expensive as well as inexpensive medium.

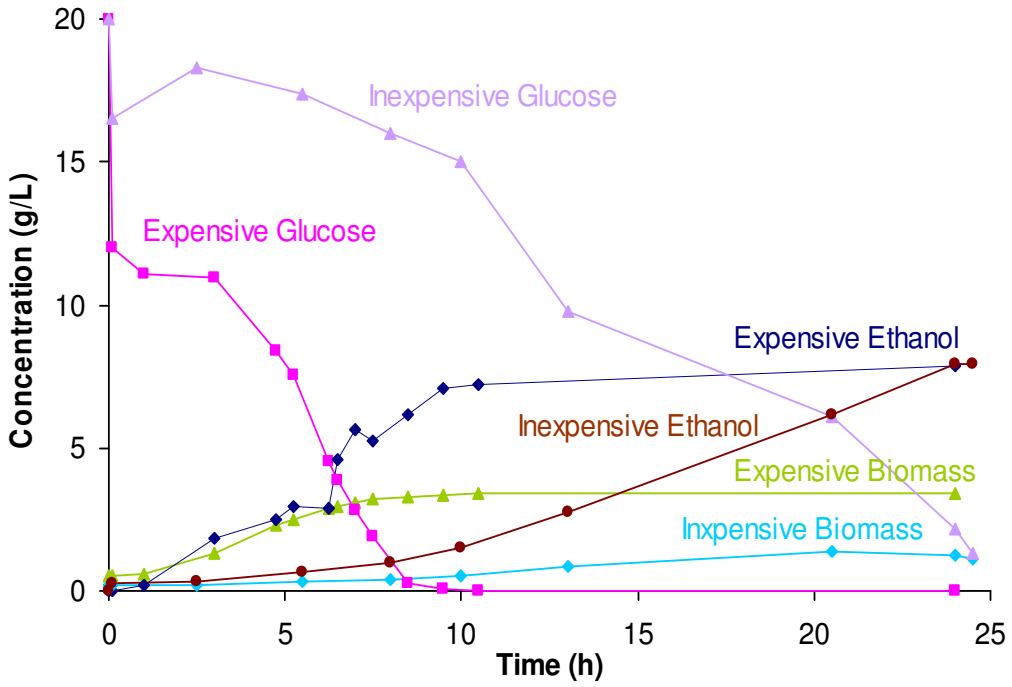


Figure 4.11: Comparison of biomass, glucose and ethanol concentration trends for Muntons yeast growth in expensive and inexpensive medium, respectively, containing 20 g/L glucose at 30°C

As seen from Figure 4.11, the maximum biomass concentration for the run performed in expensive medium was higher than for the run performed in inexpensive medium. The maximum ethanol concentration for the 20 g/L glucose run performed with Muntons yeast in both expensive medium as well as in inexpensive medium was 7.9 g/L. So, when comparing the expensive medium and the inexpensive medium, with respect to runs performed in 20 g/L glucose, it was observed that although there was difference in the amount of biomass produced, the inexpensive medium worked as well as the expensive medium and it satisfied all of the growth and ethanol production requirements for Muntons yeast.

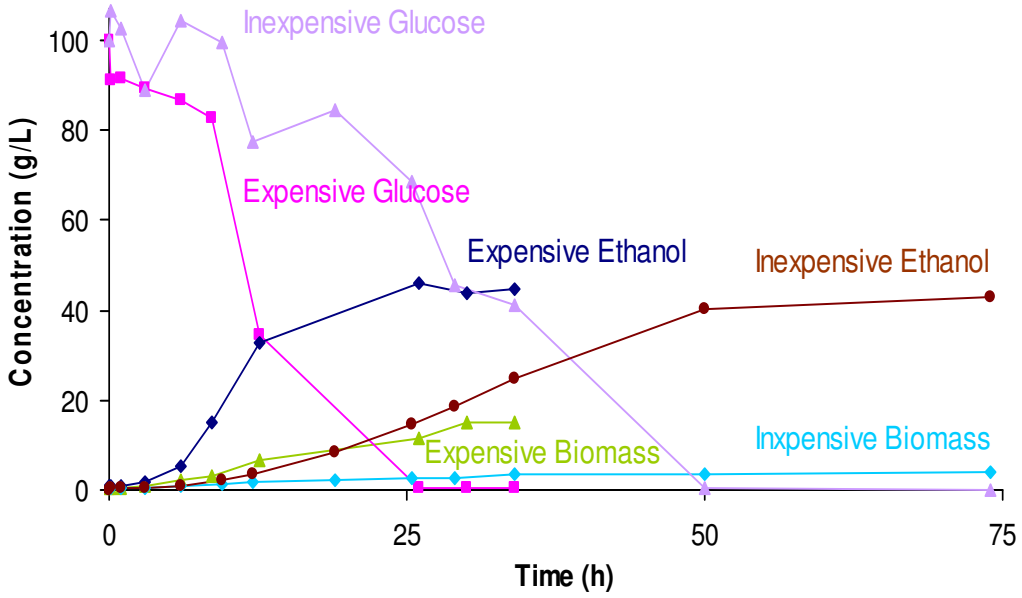


Figure 4.12: Comparison of biomass, glucose and ethanol concentration trends for Muntons yeast growth in expensive and inexpensive medium, respectively, containing 100 g/L glucose at 30°C

From Figure 4.12, again, it can be observed that the maximum biomass concentration for the run performed in expensive medium was higher than that for the run performed in inexpensive medium. The maximum ethanol concentrations for the 100 g/L glucose runs performed with Muntons yeast in expensive medium and in inexpensive medium were 44.5 g/L and 43.2 g/L, respectively. So, when comparing the expensive medium and the inexpensive medium, with respect to runs performed in 100 g/L glucose, it was observed that although there was a difference in the amount of biomass produced, the maximum ethanol concentrations for both runs were almost the same. Therefore, it can be concluded again that the inexpensive medium performed as well as the expensive medium with respect to ethanol production using Muntons yeast in 100 g/L glucose.

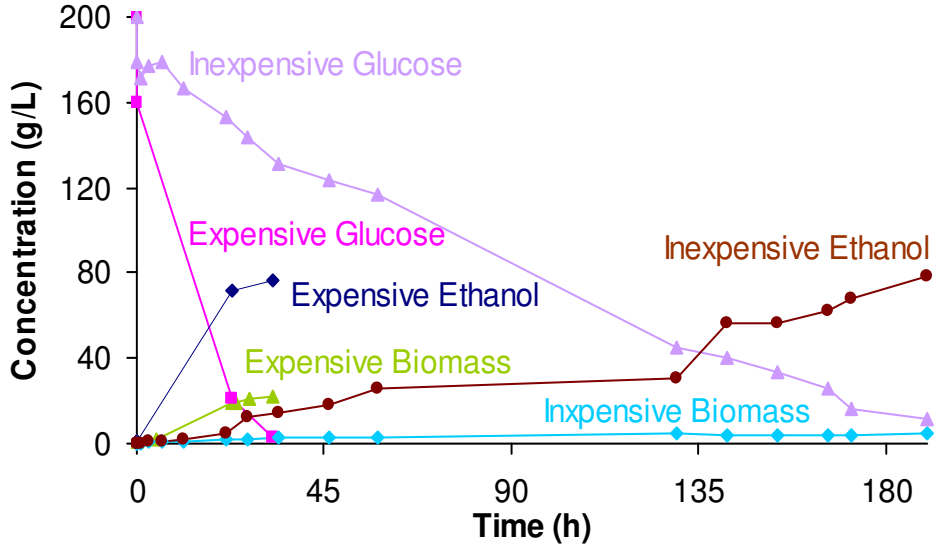


Figure 4.13: Comparison of biomass, glucose and ethanol concentration trends for Muntons yeast growth in expensive and inexpensive medium, respectively, containing 200 g/L glucose at 30°C

It can be observed from Figure 4.13, that the maximum biomass concentration for the run performed in expensive medium was higher than that for the run performed in inexpensive medium. The maximum ethanol concentrations for the 200 g/L glucose runs performed with Muntons yeast in expensive medium and in inexpensive medium were 76.4 g/L and 78.75 g/L, respectively. So, when comparing the expensive medium and the inexpensive medium, with respect to runs performed in 200 g/L glucose, it was observed that although there was a difference in the maximum biomass produced, the maximum ethanol concentrations were almost the same for both the runs. Therefore, it can be concluded that, like the 20 and 100 g/L glucose runs, the novel, inexpensive medium performed as well as the expensive medium even in 200 g/L glucose.

From Figures 4.11, 4.12 and 4.13, it can be observed that as the glucose concentration increased from 20 g/L to 200 g/L, the time required for the inexpensive medium runs to complete also increased. For instance, for the 20 g/L inexpensive medium run, almost all of the glucose was consumed in approximately 24 hours. But for the 100 g/L and 200 g/L inexpensive medium runs, glucose consumption took approximately 50 and 190 hours, respectively. On the other

hand, the expensive medium runs containing 20, 100 and 200 g/L glucose took approximately 24, 30 and 34 hours, respectively. Therefore, it can be concluded that although the novel, inexpensive medium was as good as the expensive medium at all three glucose concentrations, with respect to ethanol production, it may not be very practical to use for high glucose concentrations due to time constraints.

Table 4.5 shows the summary of the results of batch runs performed using Muntons yeast in expensive as well as inexpensive medium.

Table 4.5: Summary of glucose runs with Muntons yeast in expensive and inexpensive media

	MUNTONS YEAST WITH EXPENSIVE MEDIUM	**MUNTONS YEAST WITH INEXPENSIVE MEDIUM**
Glucose conc. (g/L)	20	20
μ (h^{-1})	0.32	0.12
Ethanol Yield (g ethanol/g glucose)	0.40	0.42
Final Glucose conc. (g/L)	0	1.3
Biomass Yield (g yeast/g glucose)	0.15	0.055
Glucose conc. (g/L)	100	100
μ (h^{-1})	0.19	0.094
Ethanol Yield (g ethanol/g glucose)	0.45	0.43
Final Glucose conc. (g/L)	0.5	0
Biomass Yield (g yeast/g glucose)	0.14	0.027
Glucose conc. (g/L)	200	200
μ (h^{-1})	0.13	0.11
Ethanol Yield (g ethanol/g glucose)	0.39	0.42
Final Glucose conc. (g/L)	2.6	11.0
Biomass Yield (g yeast/g glucose)	0.10	0.020

From Table 4.5, it is observed that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for Muntons yeast in expensive medium were 0.32, 0.19 and 0.13 h^{-1}, respectively, while those for Muntons yeast in inexpensive medium were 0.12, 0.094 and 0.11 h^{-1}, respectively. Hence, at all three glucose concentrations, the maximum specific growth rates were higher for runs performed in expensive medium as compared to runs performed in inexpensive medium. The biomass yields were higher for runs performed using the expensive medium as compared to those performed using the inexpensive medium. But, more important than the maximum specific growth rates, are the ethanol yields. At all three glucose concentrations, the ethanol yields for runs performed using the inexpensive medium (0.42, 0.43 and 0.42 g ethanol / g glucose) were comparable to those for runs performed using the expensive medium (0.40, 0.45 and 0.39 g ethanol / g glucose). Hence, it can be concluded that the novel, inexpensive medium was as good as the expensive medium when it comes to ethanol production and that it can be used for ethanol production from glucose with high ethanol yields.

4.3.4 Modelling

All the batch runs that were performed using 20 g/L and 100 g/L glucose as substrate were modelled based on the Monod equation. The Excel spreadsheets for the modelling data are attached in Appendix A. Also, for all of the glucose runs, the linear data for each run was used to plot 'ln biomass vs time' and linear regression was used to calculate the maximum specific growth rates (μ_{max}). The two methods for determining μ_{max} are compared in this section.

The following is a brief description of the Monod model:

$$\mu_g = \mu_m S / (K_S + S) \qquad (1)$$

where μ_m is the maximum specific growth rate when $S >> K_S$ and K_S, the saturation constant, is equal to the concentration of the rate-limiting substrate when the specific growth rate is equal to half of the maximum. In other words, $K_S = S$ when $\mu_g = ½ \mu_{max}$.

The Monod equation is based on the idea that substrate uptake is determined by a single enzyme system governed by Michaelis-Menten kinetics. It is assumed that the amount of that enzyme is low and hence the enzyme governs the growth-rate limiting step. The Monod equation fits a wide range of data and is the most commonly applied unstructured, non-segregated model of microbial growth (Shuler and Kargi, 2005).

Figure 4.14 shows an example of the graph of the modelled data. It highlights the fact that the model is appropriate for this data.

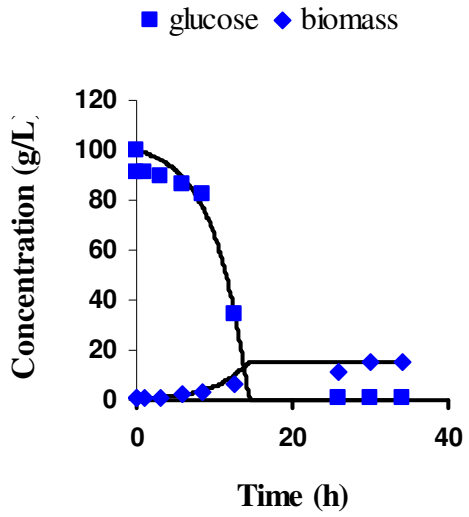


Figure 4.14: The modeled graph of the 100 g/L glucose run using Muntons yeast

Table 4.6 shows a summary of the μ_{max} values derived from the model, for all of the glucose runs.

Table 4.6: Summary of μ_{max} values obtained from modeling the data for batch runs

Glucose Concentration (g/L)	NRRL Y132 in Expensive medium μ_{max} (h^{-1})	Muntons in Expensive medium μ_{max} (h^{-1})	Muntons in Inexpensive medium μ_{max} (h^{-1})
20	0.51 ± 0.00092	0.33 ± 0.0019	0.10 ± 0.0013
100	0.32 ± 0.00076	0.24 ± 0.0022	0.050 ± 0.00094

From Table 4.6, it was observed that as the glucose concentration increased from 20 g/L to 100 g/L, the maximum specific growth rate decreased for all of the three cases; NRRL Y132 in expensive medium, Muntons in expensive medium and Muntons in inexpensive medium. The decline in the maximum specific growth rates may be due to the increase in ethanol concentrations. The K_S value was set at 2.0 g/L for all of the runs (Hill and Robinson, 1990).

Also the error numbers shown in Table 4.6 are the 'standard error of the mean' values. The 200 g/L runs were not modelled as at such high glucose concentrations, the Monod model does not fit well. At such high glucose concentrations, ethanol inhibition occurs and in order to obtain appropriate values of μ_{max}, inhibition must be included in the model (Hill and Robinson, 1990).

When comparing the NRRL Y132 strain of yeast with Muntons yeast, it was observed that the maximum specific growth rates for the NRRL Y132 yeast were higher than those of Muntons yeast. Also, when comparing the expensive medium with the inexpensive medium, it was observed that the growth rates were significantly lower for runs performed in the inexpensive medium.

Table 4.7: Summary of μ_{max} values obtained from log biomass vs time graphs for the batch runs

Glucose Concentration (g/L)	NRRL Y132 in Expensive medium μ_{max} (h^{-1})	Muntons in Expensive medium μ_{max} (h^{-1})	Muntons in Inexpensive medium μ_{max} (h^{-1})
20	0.42 ± 0.37	0.32 ± 0.06	0.12 ± 0.06
100	0.37 ± 0.40	0.19 ± 0.10	0.094 ± 0.12
200	0.41 ± 0.30	0.13 ± 0.22	0.11 ± 0.07

Comparing Tables 4.6 and 4.7, the most significant observation is that the standard errors were much higher for the results in Table 4.7 as compared to those in Table 4.6. The modelled data are more accurate and has lower error due to higher number of data points. When determining the μ_{max} by linear regression, a few data points that follow a linear trend were selected. But for modeling, all of the data points for each run were used and the sum of errors minimized, to determine the μ_{max}.

4.4 Recombinant Cell Culture on Soluble Starch

Five batch runs were performed using expensive medium and 20 g/L soluble starch as substrate. The first run was performed with the wild-type NRRL Y132 cells using only α-amylase and the second run was performed using only glucoamylase. The third run was performed using wild-type NRRL Y132 cells with both α-amylase and glucoamylase, followed by the fourth run using recombinant NRRL Y132 cells with glucoamylase alone. The third and the fourth runs were expected to produce similar results. Since the maximum ethanol produced in the fourth run was significantly lower than that produced in the third run, an additional run, run 5, was performed using recombinant NRRL Y132 cells with both α-amylase and glucoamylase. The summary is shown in Table 4.8.

Figures 4.15 and 4.16 show run 3 and 5, respectively, i.e. the production of biomass and ethanol with time during the culturing of wild-type and recombinant NRRL Y132 cells, respectively, in 20 g/L soluble starch expensive medium, containing both α-amylase and glucoamylase.

Comparing Figures 4.15 and 4.16, it was observed that although the biomass produced was similar, the maximum ethanol produced was lower for the recombinant cells as compared to the wild-type cells.

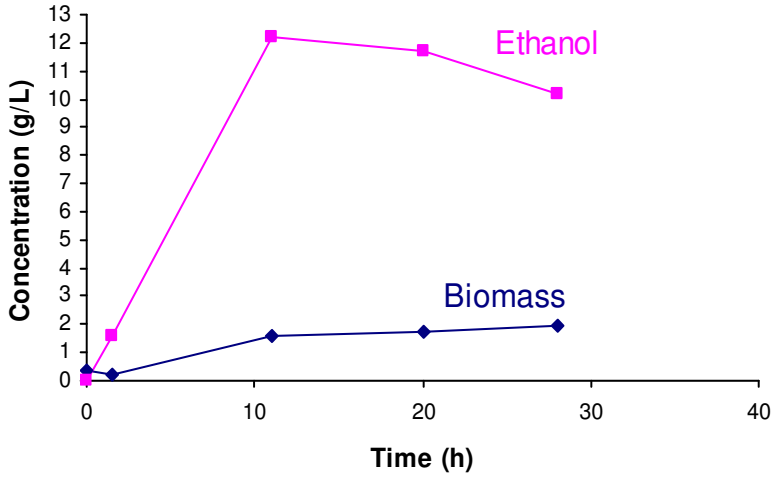


Figure 4.15: Run 3: Biomass and ethanol production curves for batch experiments at 30°C using wild-type NRRL Y132 cells in expensive medium containing 20 g/L soluble starch, α-amylase and glucoamylase

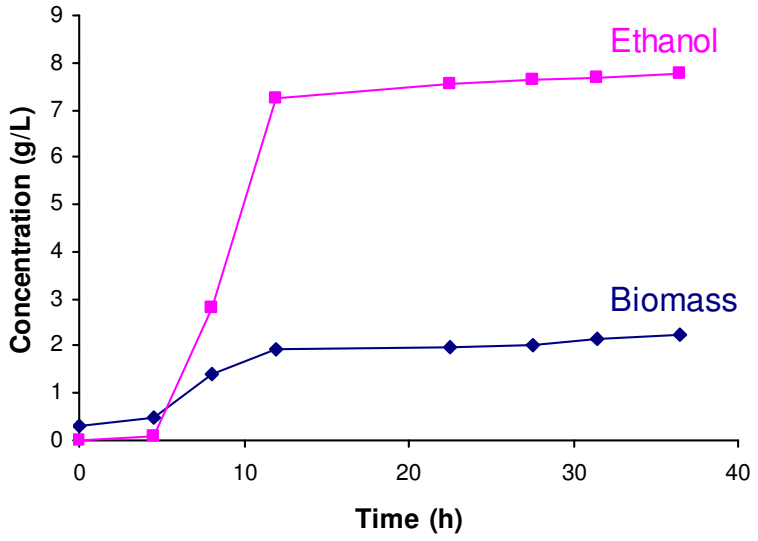


Figure 4.16: Run 5: Biomass and ethanol production curves for batch experiments at 30°C using recombinant NRRL Y132 cells in expensive medium containing 20 g/L soluble starch, α-amylase and glucoamylase

Figure 4.17 shows run 4, i.e. biomass and ethanol production curves for the run performed using recombinant NRRL Y132 cells with glucoamylase alone, in expensive medium containing 20 g/L soluble starch.

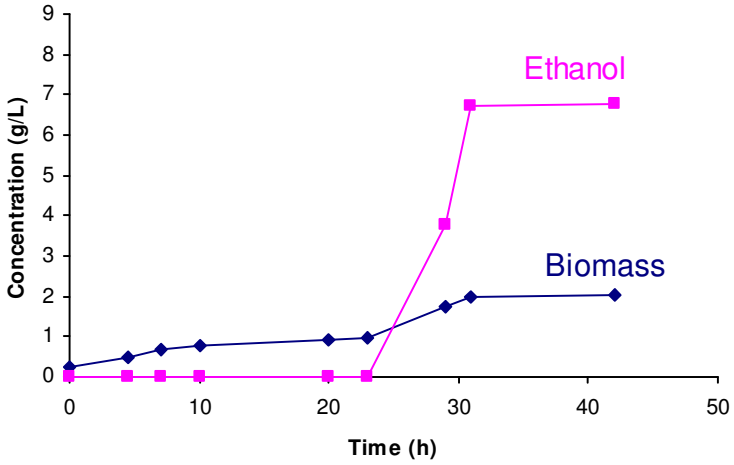


Figure 4.17: Run 4: Biomass and ethanol production curves for batch experiments at 30°C using recombinant NRRL Y132 cells in expensive medium containing 20 g/L soluble starch and glucoamylase

The results shown in Figure 4.17 were expected to be similar to those shown in Figure 4.15. But it was observed from Figure 4.17 that the maximum ethanol produced in the run shown in Figure 4.17 was lower than that shown in Figure 4.15. Also, as seen in Figure 4.17, in the run using recombinant cells with glucoamylase alone, the ethanol production started after approximately 24 hours. This may mean that the recombinant cells took some time to adapt to the environment before they were able to secrete α-amylase and convert starch to ethanol. However, it was noticeable that similar quantity of ethanol was produced as for the case when α-amylase was provided (Figure 4.16).

Table 4.8 shows a summary of the maximum specific growth rates (μ) and the maximum ethanol produced for all the batch runs performed with soluble starch as substrate.

Table 4.8: Summary of 20 g/L soluble starch batch runs

Soluble Starch Runs	μ (h^{-1})	Maximum Ethanol Concentration (g/L)
Wild-type NRRL Y132 + α-amylase	0.18	-
Wild-type NRRL Y132 + glucoamylase	0.076	6.09
Wild-type NRRL Y132 + α-amylase + glucoamylase	0.20	11.7
Recombinant NRRL Y132 + glucoamylase	0.054	6.77
Recombinant NRRL Y132 + α-amylase + glucoamylase	0.17	7.86

Comparing Table 4.8 with Table 4.4, it was observed that the growth rates of both wild-type cells and recombinant cells were significantly lower on soluble starch than those observed on glucose. Also, some of the ethanol analysis results obtained were unexpected. For instance, it was expected that the first and second runs as shown in Table 4.8 should produce negligible amounts of ethanol, since α-amylase or glucoamylase alone is not expected to breakdown soluble starch to significant amounts of glucose. But the fact that the cells grew, signifies that NRRL Y132 cells probably are capable of fermenting maltose. Grylls and Harrison demonstrated that certain strains of Baker's yeast (*S. cerevisiae*) are capable of fermenting maltose (Grylls and Harrison, 1956).

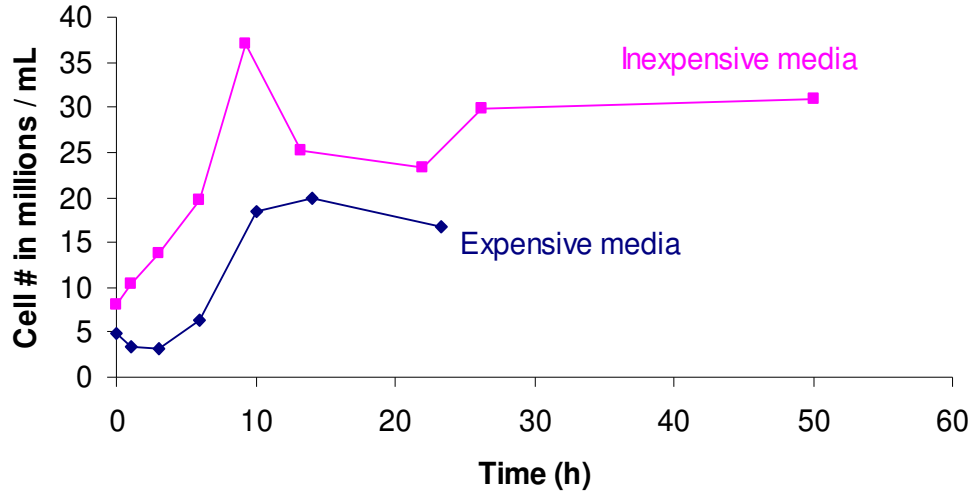
Moreover, since the recombinant cells were designed to secrete α-amylase, the fourth run in the above table was expected to produce similar amounts of ethanol as that in the third run. But the ethanol produced in runs using recombinant cells was low. As such, it was decided to focus further research on improving Muntons yeast performance on inexpensive medium and starch particles and to terminate the study of the new recombinant yeast strain, since it clearly was not capable of efficiently producing ethanol without the addition of α-amylase.

4.5 Production of Ethanol from Starch Particles

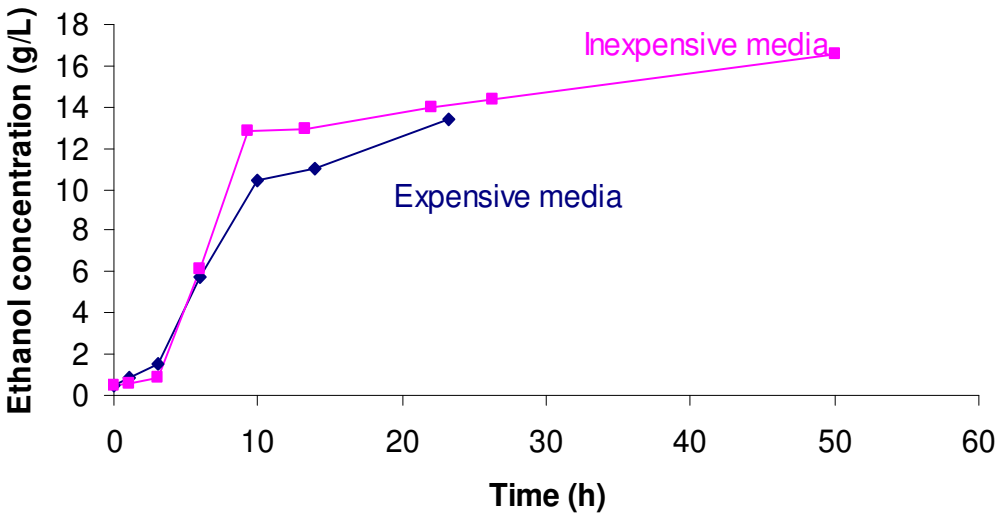
4.5.1 Expensive Medium vs Inexpensive Medium

Muntons active brewing yeast was cultured in the bioreactor at starch particle concentrations of 20, 100 and 200 g/L in both expensive and inexpensive medium. These batch runs were performed at 30°C. Figures 4.18, 4.19 and 4.20 show the biomass and ethanol concentration graphs for these batch runs.

From Figures 4.18 to 4.20, it can be observed that the biomass and the ethanol concentration trends for the expensive medium runs were comparable to those of the inexpensive medium runs. The maximum ethanol concentrations were also similar. Hence, it can be concluded that the inexpensive medium was as good as the expensive medium and that it can be used for ethanol production from starch particles.

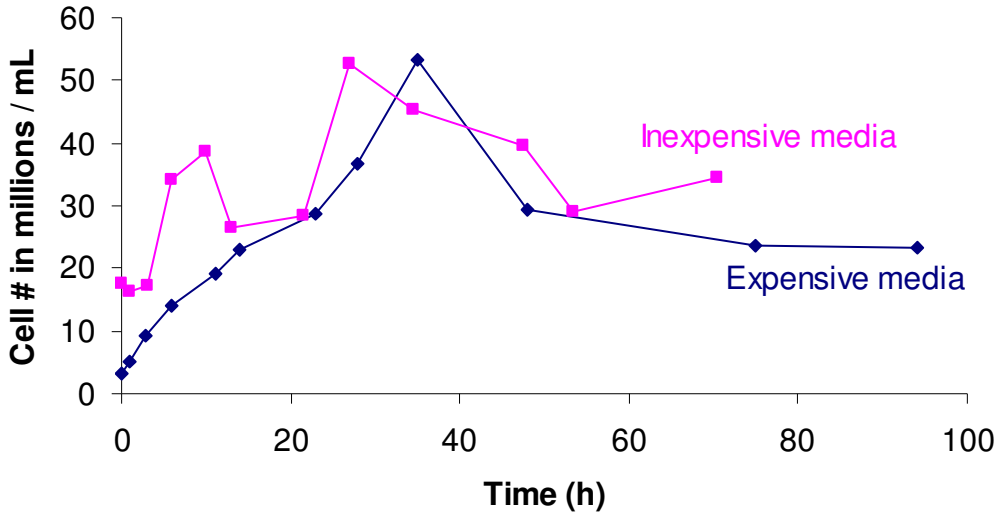


(a)

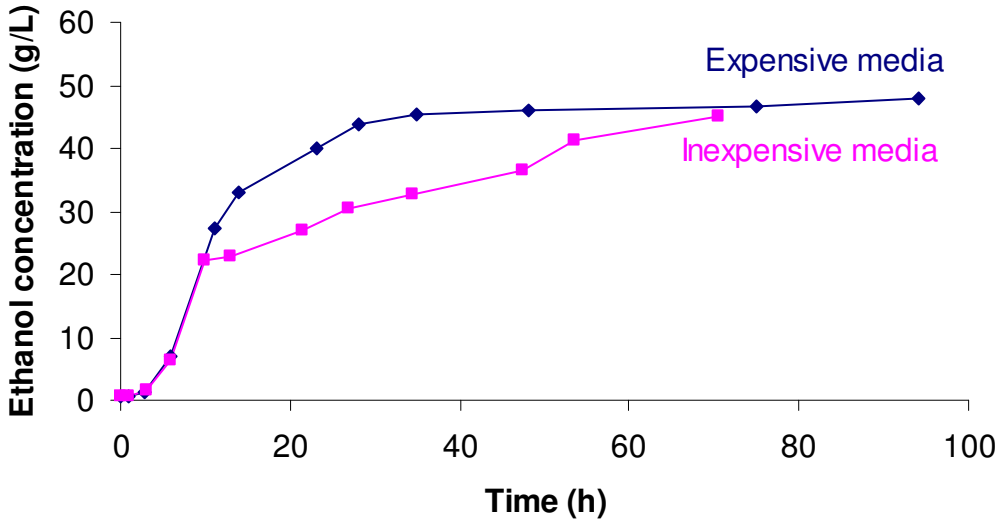


(b)

Figure 4.18: Batch growth dynamics of Muntons yeast in medium containing 20 g/L starch particles, α-amylase / barley malt and glucoamylase (a) biomass trends and (b) ethanol trends

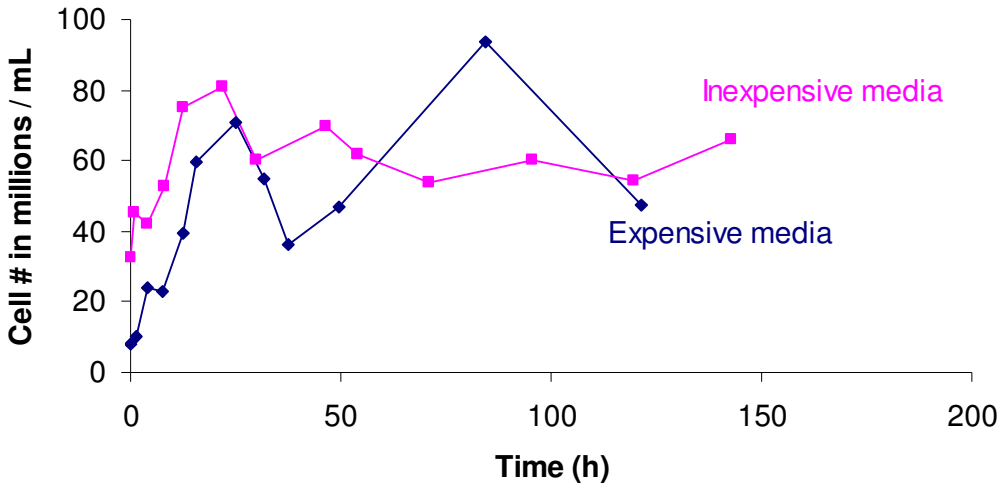


(a)

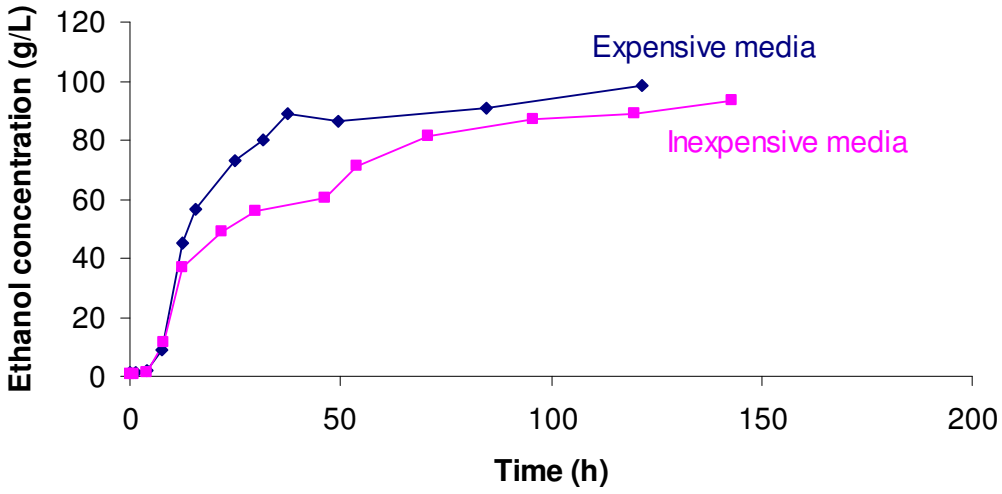


(b)

Figure 4.19: Batch growth dynamics of Muntons yeast in medium containing 100 g/L starch particles, α-amylase / barley malt and glucoamylase (a) biomass trends and (b) ethanol trends



(a)



(b)

Figure 4.20: Batch growth dynamics of Muntons yeast in medium containing 200 g/L starch particles, α-amylase / barley malt and glucoamylase (a) biomass trends and (b) ethanol trends

Figure 4.21 shows the decreasing trends of total dry solids' concentrations for the three inexpensive medium runs performed at 30°C. The difference between the initial and final total dry solids' concentrations was used to determine the ethanol yields for the inexpensive medium runs.

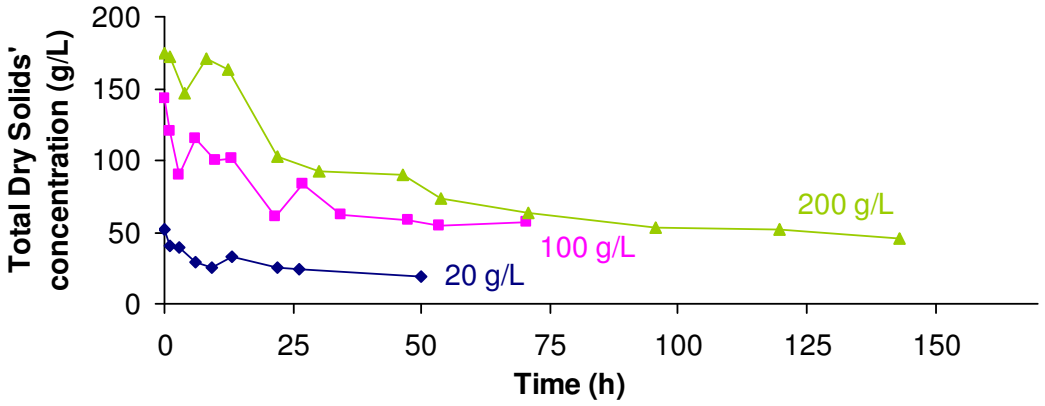


Figure 4.21: Total dry solid concentration trends for the three inexpensive medium runs at 30°C shown in Figures 4.18, 4.19 and 4.20

Table 4.9 shows a summary of the initial and final starch concentrations for the three expensive medium runs. These starch concentrations were measured using the iodine method (Xiao *et al*., 2006). The difference between the initial and final concentrations was used to calculate the ethanol yields for each of the three runs.

Table 4.9: Summary of Initial and Final Starch concentrations

	Initial Starch concentration (g/L)	Final Starch concentration (g/L)
20 g/L	26.5	3.3
100 g/L	123.9	5.3
200 g/L	209.2	9.2

Table 4.10 shows a summary of the results of batch runs performed using Muntons yeast in both expensive medium and inexpensive medium containing 20, 100 and 200 g/L starch particles.

Table 4.10: Summary of starch particle runs using expensive and inexpensive media

	EXPENSIVE MEDIUM	INEXPENSIVE MEDIUM
Starch particles (g/L)	20	20
μ (h^{-1})	0.25	0.16
Ethanol Yield (g ethanol/g substrate)	0.41	0.46
Starch particles (g/L)	100	100
μ (h^{-1})	0.13	0.11
Ethanol Yield (g ethanol/g substrate)	0.38	0.44
Starch particles (g/L)	200	200
μ (h^{-1})	0.12	0.068
Ethanol Yield (g ethanol/g substrate)	0.42	0.43

From Table 4.10, it was observed that at starch particle concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the runs performed in expensive medium were 0.25, 0.13 and 0.12 h^{-1} while those for runs performed in inexpensive medium were 0.16, 0.11 and 0.068 h^{-1}. Hence, at all three starch particle concentrations, the maximum specific growth rates were higher for the runs performed in expensive medium as compared to those performed in the novel, inexpensive medium. But at all three starch particle concentrations, the ethanol yields were slightly higher for runs performed in inexpensive medium (0.46, 0.44 and 0.43 g ethanol / g total dry solids') as compared to those performed in expensive medium (0.41, 0.38 and 0.42 g ethanol / g glucose). Therefore, it can be concluded that the novel, inexpensive medium was as good as the expensive medium and that it can be used for ethanol production from starch particles with good ethanol yields.

4.5.2 Sequential Hydrolysis and Fermentation

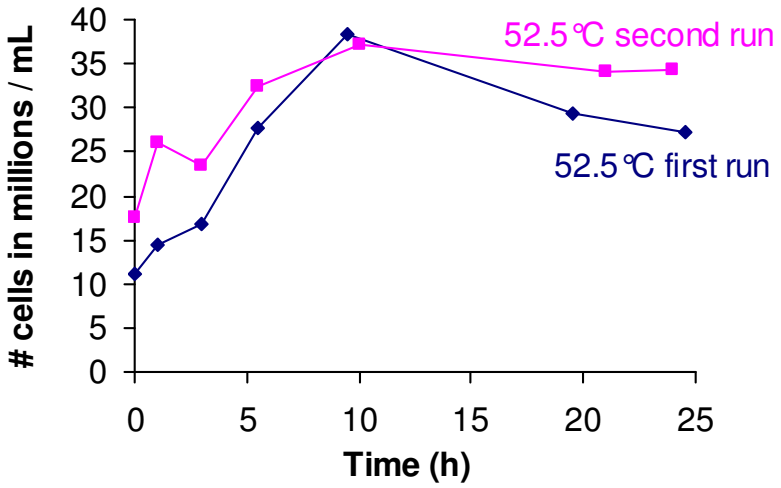
Three sequential hydrolysis and fermentation runs were carried out using Muntons yeast in inexpensive medium containing 100 g/L starch particles. The hydrolysis of starch particles was carried out for a period of three hours at 37.5°C, 45.0°C and 52.5°C, respectively and the actual fermentations were then performed at 30°C. The graphs for these runs are presented and discussed in section 4.5.3. Textor *et al*. in 1998 performed hydrolysis of wheat starch granules and found that barley α-amylase at pH 4.5 and at 45°C was the most efficient. At starch and enzyme concentrations of 30 g/L and 8 g/L, respectively, barley α-amylase was able to hydrolyse 98% of the starch granules in three hours (Textor *et al*., 1998). The 52.5°C sequential hydrolysis and fermentation run was performed two times to test reproducibility.

Table 4.11 shows a summary of the four sequential hydrolysis and fermentation runs. As observed from the table, both the maximum specific growth rates and the ethanol yields were almost the same in spite of the three different hydrolysis temperatures. For the 52.5°C hydrolysis, the maximum specific growth rate mentioned in Table 4.11 is the average of the values obtained from the two runs.

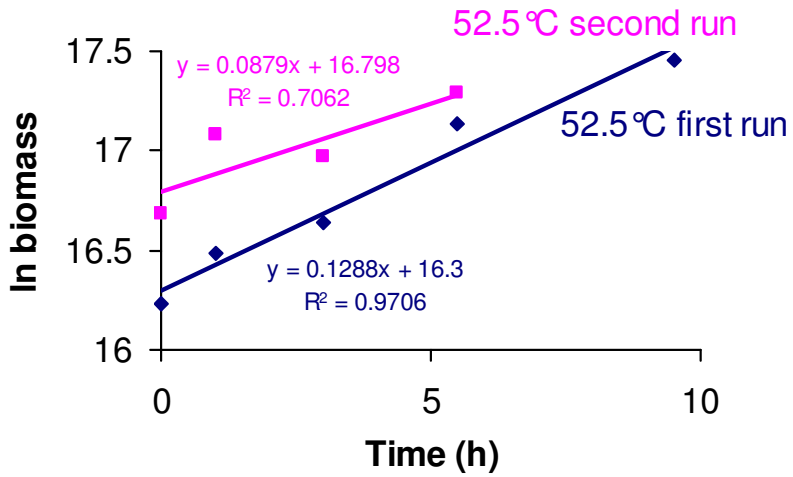
Table 4.11: Summary of sequential hydrolysis and fermentation runs

	30°C HYDROLYSIS	**37.5°C HYDROLYSIS**	**45°C HYDROLYSIS**	**52.5°C HYDROLYSIS**
Starch particles (g/L)	100	100	100	100
μ (h^{-1})	0.11	0.10	0.10	0.11
Ethanol Yield (g ethanol/g total dry solids')	0.44	0.37	0.44	0.39

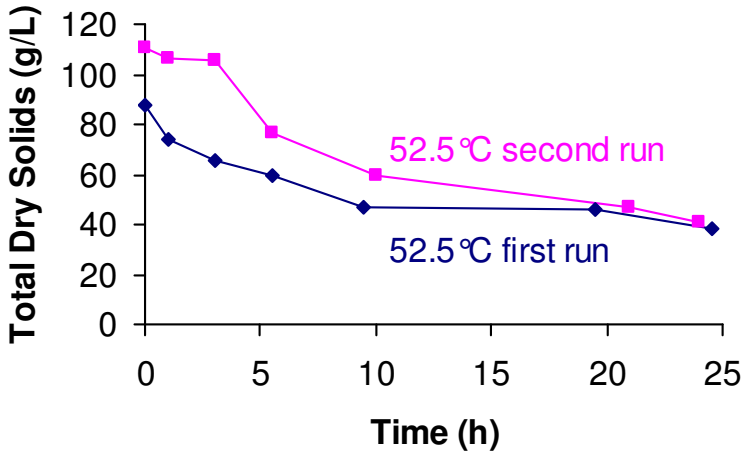
The results of the two 52.5°C runs are presented below. Figures 4.22 (a), (b) and (c) show the biomass, natural logarithm of biomass and total dry solids versus time results, respectively, for the two 52.5°C runs.


40
35
30
25
20
15
10
5
0
cells in millions / mL
0
5
10
15
20
25
Time (h)
52.5℃ second run
52.5℃ first run

(a)


17.5
17
16.5
16
ln biomass
0
5
10
Time (h)
52.5℃ second run
y = 0.0879x + 16.798
R² = 0.7062
52.5℃ first run
y = 0.1288x + 16.3
R² = 0.9706

(b)



(c)

Figure 4.22: Comparison of two sequential hydrolysis and fermentation run results performed using Muntons yeast at a hydrolysis temperature of 52.5°C, containing 100 g/L starch particles, barley malt and glucoamylase (a) biomass concentration trends, (b) ln biomass trends and (c) total dry solid concentration trends

As observed from the above graphs, the two 52.5°C runs had similar results. The 'biomass versus time' curves had almost the same pattern in spite of having different maximum biomass concentrations. Also the maximum specific growth rates were 0.13 h^{-1} and 0.088 h^{-1} for the first and second run, respectively. The decreasing trends of total dry solids with time for both the runs were similar and the final total dry solid concentration was also almost the same, 38.3 g/L for the first run and 40.7 g/L for the second run. Although the ethanol data was not obtained, it was still quite clear that the sequential hydrolysis and fermentation run at 52.5°C was reproducible.

4.5.3 Effect of Temperature on Hydrolysis of Starch Particles

The three runs as mentioned in section 4.5.2 were performed using the novel, inexpensive medium containing 100 g/L starch particles with pre-hydrolysis carried out at 37.5°C, 45.0°C and 52.5°C, respectively. Both α-amylase, from the malt and Valley Research glucoamylase enzymes were used to breakdown the starch particles.

Figures 4.23, 4.24, 4.25 and 4.26 represent a comparison of biomass, natural logarithm of biomass, total dry solids' and ethanol concentration trends in approximately the first 25 hours of the runs performed at 30°C, 37.5°C, 45°C and 52°C, respectively.

Figure 4.23 depicts the fact that although the biomass concentrations were not exactly the same for the four runs performed at different temperatures, the shape of all four graphs was similar. This shows that the pattern of growth of the yeast cells was the same and using elevated temperatures to improve the hydrolysis rates, did not improve the subsequent fermentation process. Figure 4.24 shows that the maximum specific growth rates were almost the same for all four runs and hence, increasing the temperature to improve hydrolysis did not trigger the yeast cells to grow faster. The decreasing trend in total dry solids and the increasing trend in ethanol concentrations was also the same for all four temperatures.

As observed from Figure 4.26, after approximately 25 hours, the 52.5°C run had the maximum ethanol produced, as compared to the other three. It is known that at approximately 55°C-60°C, starch particles begin to swell in aqueous solution (Archer Daniels Midland brochure, undated). But if we consider the data for both the 52.5°C runs, the average maximum specific growth rate was 0.11 h^{-1}, which was same as the maximum specific growth rate at the other lower temperatures. Therefore, it can be concluded that increasing the temperature above 30°C for enhancing the hydrolysis of starch did not seem to increase fermentation productivity significantly.

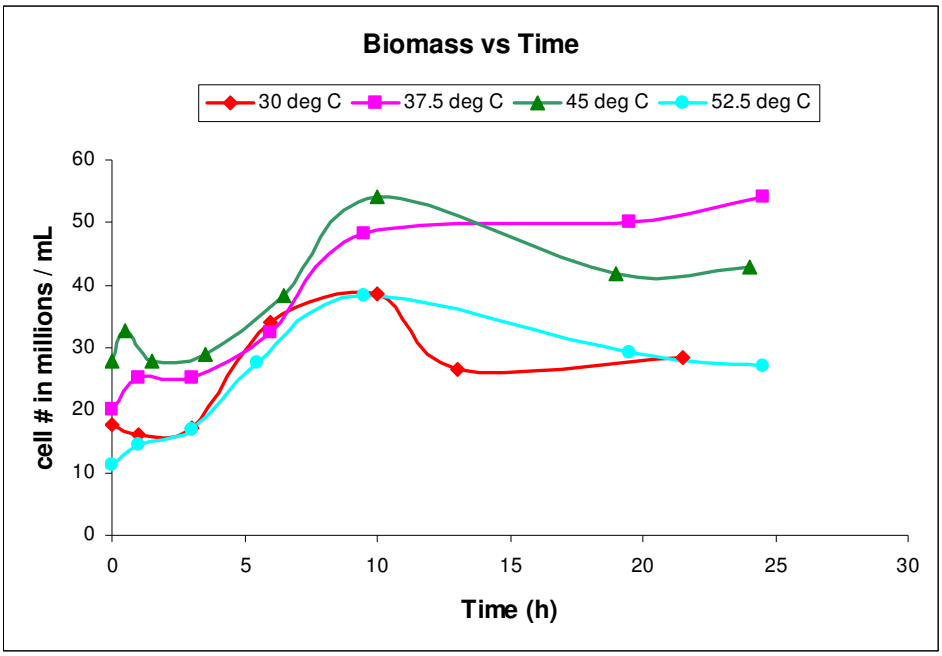


Figure 4.23: Biomass production curves for batch experiments using Muntons yeast in novel, inexpensive medium containing 100 g/L starch particles, barley malt and glucoamylase

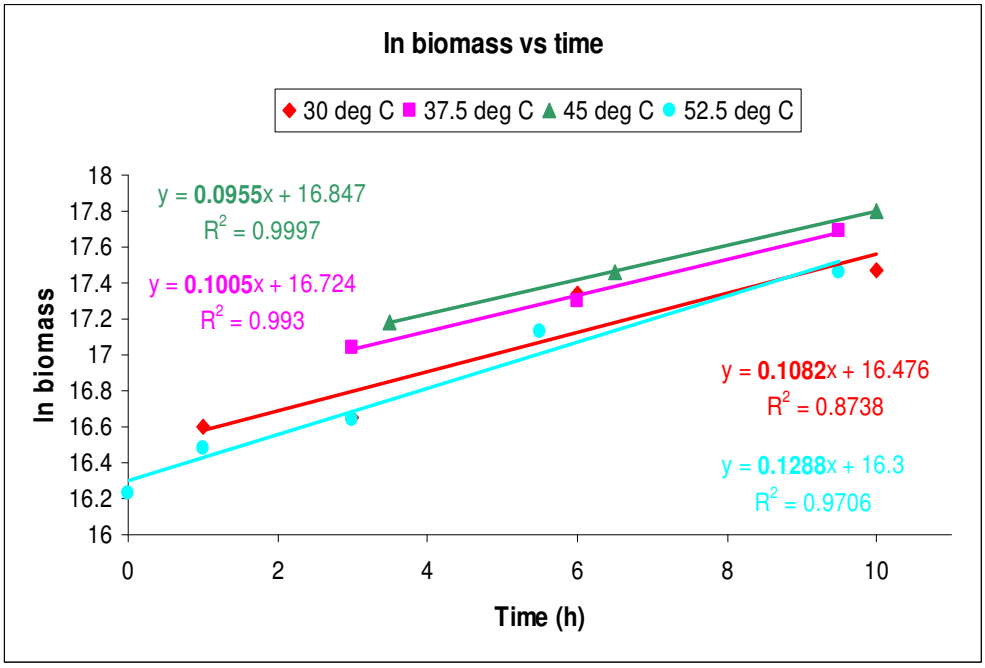


Figure 4.24: Natural logarithm of biomass curves for batch experiments using Muntons yeast in novel, inexpensive medium containing 100 g/L starch particles, barley malt and glucoamylase

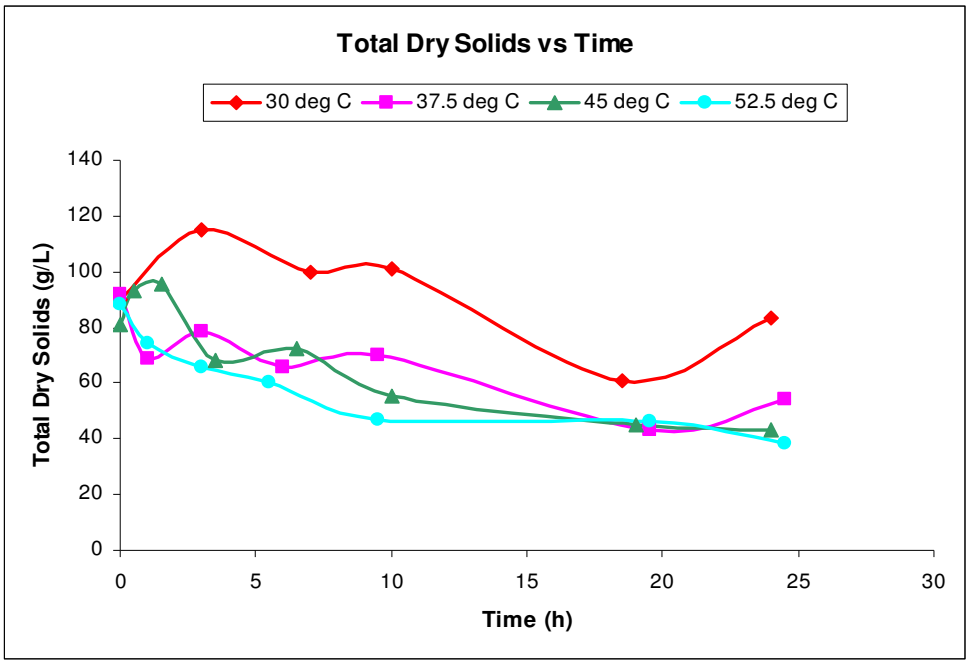


Figure 4.25: Total dry solids vs time curves for batch experiments using Muntons yeast in novel, inexpensive medium containing 100 g/L starch particles, barley malt and glucoamylase

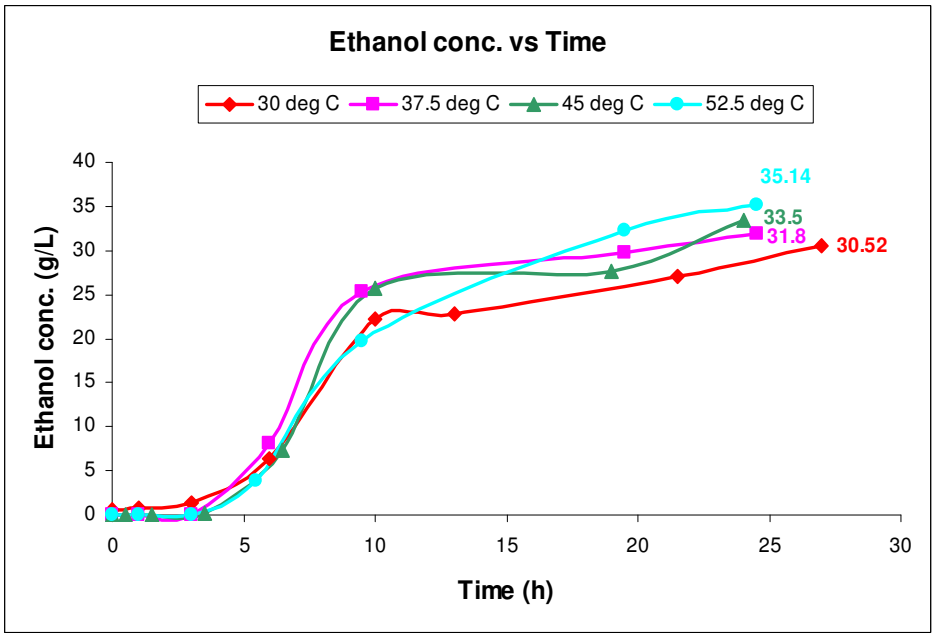


Figure 4.26: Ethanol production curves for batch experiments using Muntons yeast in novel, inexpensive medium containing 100 g/L starch particles, barley malt and glucoamylase

Table 4.12 shows a summary of the maximum specific growth rates (μ) and the ethanol yields for the batch runs performed with starch particles as substrate. The novel, inexpensive medium runs with starch particles as substrate gave comparable results to those achieved with expensive medium. The ethanol yields were comparable to those obtained from the expensive medium runs. Hence, it can be concluded that the novel, inexpensive medium was as good as the expensive medium and can be used for ethanol production with high yields. Moreover, the use of starch particles as substrate avoids the high temperature cooking procedure, thereby, making this entire fermentation process for bioethanol production more efficient than current technology used in the bioethanol industry in North America.

Table 4.12: Summary of starch particle batch runs

	EXPENSIVE (30°C)	INEXPENSIVE (30°C)	INEXPENSIVE (37.5°C)***	INEXPENSIVE (45°C)***	INEXPENSIVE (52.5°C)***
Starch particles (g/L)	20	20	-	-	-
μ (h^{-1})	0.25	0.16	-	-	-
Ethanol Yield **	0.41	0.46	-	-	-
Starch particles (g/L)	100*	100	100	100	100
μ (h^{-1})	0.094	0.11	0.10	0.10	0.11
Ethanol Yield**	0.38	0.44	0.37	0.44	0.39
Starch particles (g/L)	200	200	-	-	-
μ (h^{-1})	0.12	0.068	-	-	-
Ethanol Yield**	0.42	0.43	-	-	-

* The 100g/L expensive medium run was performed three times. The μ_{max} is 0.094 ± 0.03 h^{-1}, the ethanol yield is 0.38 ± 0.04 g ethanol / g glucose and the maximum ethanol concentration is 46.9 ± 1.3 g/L. There is a ±13.9 % error in the counting of cell numbers for biomass measurements and the error in the measurement of total dry solids is ±8.6%.

** The ethanol yields are calculated by dividing the maximum ethanol produced by the difference in initial and final total dry solids.

*** The temperatures 37.5°C, 45°C and 52.5°C are hydrolysis temperatures. The fermentation was performed at 30°C for all the runs.

5.0 Conclusions and Recommendations

5.1 Summary and Conclusions

- When comparing the two types of yeast – *Saccharomyces cerevisiae* strain NRRL Y132 and Muntons Active Brewing yeast, with respect to runs performed using glucose as substrate, it was observed that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the NRRL Y132 yeast (0.42, 0.37 and 0.41 h^{-1}, respectively) were significantly higher than those of Muntons yeast (0.32, 0.19 and 0.13 h^{-1}, respectively). Also, at all three glucose concentrations, the ethanol yields were slightly higher for runs performed with the NRRL Y132 yeast as compared to those performed with Muntons yeast. However, since there were only slight differences in the ethanol yields, it may be concluded that with respect to the efficiency of ethanol production, both types of yeast are equally good.

- When comparing the recombinant and wild-type NRRL Y132 yeast cells with respect to runs performed using glucose as substrate, it was observed that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the wild-type NRRL Y132 yeast (0.42, 0.37 and 0.41 h^{-1}, respectively) were higher than those for the recombinant NRRL Y132 yeast (0.34, 0.33 and 0.24 h^{-1}, respectively). At glucose concentrations of 20 and 100 g/L, the ethanol yields were higher for runs performed with the recombinant yeast as compared to those performed with the wild-type yeast and at a glucose concentration of 200 g/L, the ethanol yields were the same for both the wild-type and the recombinant cells. Therefore, it can be concluded that the recombinant cells can grow and produce good ethanol yields on low, intermediate as well as high glucose concentrations.

- When comparing the expensive medium to the inexpensive medium with respect to runs performed using glucose as substrate, it was observed that at glucose concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for Muntons yeast in expensive medium (0.32, 0.19 and 0.13 h^{-1}, respectively) were higher than those for Muntons yeast in inexpensive medium (0.12, 0.094 and 0.11 h^{-1}, respectively). At all three glucose

concentrations, the ethanol yields for runs performed using the inexpensive medium were comparable to those for runs performed using the expensive medium. Hence, it can be concluded that the novel, inexpensive medium is as good as the expensive medium with respect to ethanol production and that it can be used for ethanol production from glucose with good ethanol yields.

- Although the novel, inexpensive medium is as good as the expensive medium with respect to ethanol production, complete utilization of glucose takes much longer when using the inexpensive medium as compared to the expensive medium. Hence, it may not be very practical to use the novel medium for high glucose concentrations due to time constraints.

- For the batch runs performed using soluble starch, the ethanol produced in runs using recombinant cells was low. It was clear that the recombinant cells were not capable of efficiently producing ethanol without the addition of α-amylase. This contradicts the purpose of the recombinant strain and so more research is needed to develop a superior strain.

- When comparing the expensive medium to the inexpensive medium with respect to runs performed using starch particles as substrate, it is observed that at starch particle concentrations of 20, 100 and 200 g/L, the maximum specific growth rates for the runs performed in expensive medium (0.25, 0.13 and 0.12 h^{-1}, respectively) are higher than those for runs performed in inexpensive medium (0.16, 0.11 and 0.068 h^{-1}, respectively). But at all three starch particle concentrations, the ethanol yields are slightly higher for runs performed in inexpensive medium as compared to those performed in expensive medium. Therefore, it can be concluded that the novel, inexpensive medium is as good as the expensive medium and that it can be used for ethanol production from starch particles with good ethanol yields.

- Increasing the temperature above 30°C for enhancing the hydrolysis of starch does not seem to increase the subsequent fermentation productivity significantly.

5.2 Recommendations

This study has demonstrated the technical suitability of a new inexpensive medium to be used in the bioethanol industries. Experiments consisting of a series of sequential batch hydrolysis and fermentation runs should be performed to test the long term stability of the simultaneous cold-starch hydrolysis and fermentation process. Two liters of the novel, inexpensive medium, containing 100 g/L starch particles, 30 g/L barley malt, 1 g/L glucoamylase and 100 mL Muntons yeast inoculum can be used. After the completion of each batch run, the yeast cells must be separated by centrifugation and the fermentation broth circulated through hollow fiber membrane ultrafilters to recover most of the aqueous phase containing ethanol. The yeast and concentrated enzyme are to be returned to the bioreactor, thereby simulating recycle flows. Fresh medium containing starch particles should be added to the bioreactor (without yeast and enzymes) such that the starch particle concentration in the bioreactor is brought back to 100 g/L and the total volume of the medium returned to 2.0 L. The procedure mentioned above is based on previous experiments performed by Lang *et al.* in 2001. They showed that four such sequential batch operations could be performed smoothly using the expensive medium, within a total time of 110 hours. It would be worthwhile to confirm that this will also be the case with the novel, inexpensive medium. When the sequential batch hydrolysis and fermentation experiments are successful, further research can focus on the development of continuous hydrolysis and fermentation of starch particles to produce ethanol, using the inexpensive medium.

For future studies of a better recombinant yeast strain, the use of two-stage CFSTR (Continuous Flow Stirred Tank Reactor) fermentation may be more efficient and productive. In 1985, Siegel and Ryu proposed the concept of a two stage continuous flow stirred tank reactor (CFSTR). They used a recombinant bacterium containing a temperature sensitive-switching gene. The first stage is called the growth stage and the second stage is the production stage during which the secretion of the gene product is induced. Some growth also occurs in the second stage to maximize gene expression and optimize product formation. A two stage fermentation system has also been found to enhance culture stability and increase productivity. Baheri *et al.* (1997) studied the effect of increasing the number of CFSTRs to greater than two and found that increasing the number of CFSTRs in the growth stage had no effect on productivity whereas increasing them in the production stage increased productivity.

Another recommendation for future studies would be the use of cellulose instead of starch as the substrate for bioethanol production. Some common cellulosic biomass resources are forest materials, wood, municipal solid wastes, waste paper and agricultural residues (Demirbaş, A., 2005). Cellulose is the main component of plant cell walls and it is the most abundant organic compound on the earth. Approximately 180 million tons of agricultural biomass per year is produced in the U.S.. Although cellulosic biomass is abundant, it is challenging to efficiently and economically produce ethanol from it. Both chemical and enzymatic methods are required to render the sugars in the cellulose accessible to conversion into ethanol. Cheaper sugar means cheaper fermentations (Novozymes and BBI International, 2005). Although economical ethanol production from fiber-based sources still requires a lot of research, with significant risks and uncertainties, we need to continue to be optimistic about its future. Iogen Corporation in Ottawa, Canada, is a biotechnology company specializing in cellulose-based enzyme technology. They operate the world's largest cellulose ethanol facility (Iogen Corporation's website). In the words of C. E. Wyman, "Cellulosic ethanol is promising because it can capitalize on the power of biotechnology to dramatically reduce costs, is derived from low cost and plentiful feedstocks, can achieve the high yields vital to success, has high octane and other desirable fuel properties, and is environmentally friendly" (Wyman, C. E., 2007).

6.0 References

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7.0 Appendices

Appendix A: Excel Spreadsheets for Modelling

(as examples, data for some of the Muntons yeast runs are presented here)

Table A.1: Muntons yeast in expensive medium containing 20 g/L glucose:

TIME, H	X EXP	XTHE	Glucose EXP	Glucose THE	Error	lnx
0	0.494571	0.494571	20	20	0	-0.704064
0.083	0.494571	0.494571	12.011572	20	63.81498	-0.704064
1	0.616345	0.665321	11.07358	18.84237299	60.35654	-0.483948
3	1.341687	1.161525	10.934044	15.47828107	20.68255	0.2939278
4.75	2.26635	1.93619	8.406892	10.22631324	3.419299	0.8181708
5.25	2.496872	2.216882	7.515412	8.32331721	0.731106	0.9150389
6.25	2.895364	2.845777	4.540582	4.05962226	0.233781	1.0631107
6.5	2.956099	3.034885	3.887476	2.777531302	1.238184	1.0838706
7	3.097411	3.300402	2.823514	0.977419691	3.449269	1.1305667
7.5	3.19162	3.426163	1.889398	0.124800049	3.168817	1.1605285
8.5	3.301579	3.444529	0.292486	0.000286975	0.105815	1.194401
9.5	3.350501	3.444571	0.096748	5.54456E-07	0.018209	1.2091099
10.5	3.424262	3.444571	0	1.07081E-09	0.000412	1.230886
24	3.435167	3.444571	0	0	8.84E-05	1.2340656
					157.2191	

EQUATIONS:

SpGr=SpGrMax*Substrate/(Ksat+Substrate)

dX/dt=SpGr*Biomass

Substrate=Sub0-(Biomass-Bio0)/YieldX

Product=Prod0+YieldP*(Sub0-Substrate)

SpGrMax	0.326904
Ksat	2
YieldX	0.1475
YieldP	0
Sub0	20
Bio0	0.494571
Prod0	0
Delt	0.1

Table A.2: Muntons yeast in expensive medium containing 100 g/L glucose:

TIME, H	X EXP	XTHE	Glucose EXP	Glucose THE	Error	lnx
0	0.509111	0.509111	100	100	0	-0.675088
0.083	0.509111	0.509111	91.1	100	79.21	-0.675088
1	0.588628	0.645577	91.5	99.05820718	57.12974	-0.529961
3	1.10238	1.013541	89.5	96.51877545	49.2711	0.0974717
6	2.185168	2.114167	86.8	88.92300892	4.512208	0.7816926
8.583333	3.114223	3.819734	82.6	77.15236585	30.17446	1.1359798
12.5833	6.553123	9.748539	34.7	36.23583586	12.56948	1.8799417
26	11.53358	14.99911	0.6	0	12.36989	2.4452629
30	14.96869	14.99911	0.5	0	0.250925	2.705961
34	15.0523	14.99911	0.5	0	0.252829	2.7115308
					245.7406	

EQUATIONS:

SpGr=SpGrMax*Substrate/(Ksat+Substrate)

dX/dt=SpGr*Biomass

Substrate=Sub0-(Biomass-Bio0)/YieldX

Product=Prod0+YieldP*(Sub0-Substrate)

SpGrMax	0.242245
Ksat	2
YieldX	0.1449
YieldP	0
Sub0	**100**
Bio0	**0.509111**
Prod0	0
Delt	0.1

Table A.3: Muntons yeast in inexpensive medium containing 20 g/L glucose:

TIME, H	X EXP	XTHE	Glucose EXP	Glucose THE	Error	lnx
0	0.165828	0.165828	20	20	0	-1.796802
2.5	0.165828	0.206166	18.3	19.2665883	0.934831	-1.472466
5.5	0.229359	0.272762	17.4	18.05575207	0.431992	-1.147999
8	0.317271	0.341322	16	16.80920584	0.660643	-0.87306
10	0.417672	0.407824	15	15.60007571	0.370241	-0.676153
13	0.50857	0.530855	9.8	13.36315487	12.79769	-0.162956
20.5	0.849628	0.975011	6.1	5.287585379	0.829916	0.327286
24	1.387198	1.200687	2.19	1.184383794	1.01477	0.2310324
24.5	1.2599	1.221633	1.28	0.8035424	0.227012	
					17.26709	

EQUATIONS:

SpGr=SpGrMax*Substrate/(Ksat+Substrate)

dX/dt=SpGr*Biomass

Substrate=Sub0-(Biomass-Bio0)/YieldX

Product=Prod0+YieldP*(Sub0-Substrate)

SpGrMax	0.099942
Ksat	2
YieldX	0.055
YieldP	0
Sub0	20
Bio0	0.165828
Prod0	0
Delt	0.1

Table A.4: Muntons yeast in inexpensive medium containing 100 g/L glucose:

TIME, H	X EXP	XTHE	Glucose EXP	Glucose THE	Error	lnx
0	0.384929	0.384929	100	100	4E-14	-0.954697
0.083	0.384929	0.384929	106.56	100	43.0336	-0.954697
1	0.491846	0.404409	102.56	99.27039452	10.82915	-0.709591
3	0.524697	0.44417	88.96	97.78121763	77.82036	-0.644934
6	0.775563	0.517574	104.32	95.03201184	86.33328	-0.254166
9.5	1.132151	0.615056	99.52	91.38100967	66.51055	0.1241195
12	1.553248	0.69567	77.44	88.36177081	120.0205	0.440348
19	2.350643	0.981547	84.64	77.65476735	50.6679	0.854689
25.5	2.556114	1.342955	68.5	64.1188946	20.66584	0.9384883
29	2.841624	1.59268	45.6	54.76588969	85.57339	1.0443756
34	3.342758	2.027686	41	38.47353588	8.112437	1.2067963
50	3.750117	3.054929	0.299	3.39639E-12	0.572687	1.321787
74	3.907207	3.054929	0.03	0	0.727278	1.3628228
141.5	3.051873	3.054929	0.017	0	0.000298	1.1157556
				0		
					570.8673	

EQUATIONS:

SpGr=SpGrMax*Substrate/(Ksat+Substrate)

dX/dt=SpGr*Biomass

Substrate=Sub0-(Biomass-Bio0)/YieldX

Product=Prod0+YieldP*(Sub0-Substrate)

SpGrMax	0.050359
Ksat	2
YieldX	0.0267
YieldP	0
Sub0	100
Bio0	0.384929
Prod0	0
Delt	0.1

Appendix B: Chemical Composition of Inexpensive Medium

The novel, inexpensive medium has four major components. They are described below:

(1) **Soluble garden fertilizer** (Plant-Prod® Fertilizer): This is a water soluble fertilizer comprised of nutritional micronutrients. A 2.0 kg container of it costs approximately $12.99. The detailed composition of this fertilizer is shown in Table B.1:

Table B.1: Composition of Soluble Fertilizer

COMPOUND	PERCENTAGE
Total Nitrogen	30
Available Phosphoric Acid	10
Soluble Potash	10
Boron	0.02
Chelated Copper	0.05
Chelated Iron	0.10
Chelated Manganese	0.05
Chelated Zinc	0.05
Molybdenum	0.0005
EDTA (chelating agent)	1

(2) **Fermaid K** (Lallemand, Quebec): This is a blended complex yeast nutrient. A 100 g packet of it costs approximately $9.50. The detailed description of its components is shown below:

Table B.2: Composition of Fermaid K

COMPONENT	COMPOUND	FUNCTION
Inorganic Nitrogen	Diammonium Phosphate	ATP, Phospholipid and Protein Synthesis
Organic Nitrogen	Alpha amino nitrogen derived from yeast extract	Protein Synthesis
Nutrients	Magnesium Sulfate	Important Co-enzyme
	Thiamine	Deficiency leads to poor growth and H_2S, acetic acid and pyruvic acid accumulation.
	Folic Acid	-
	Niacin	Cannot be synthesized by yeast cells in anaerobic conditions.
	Biotin	-
	Calcium Pantothenate	Deficiency results in higher levels of volatile acidity.
Inactivated Yeast	-	Absorb medium chain fatty acids that are toxic to yeast and also supply sterols and lipids.

(3) **Barley Malt** (InfraReady Products Pvt. Ltd.): It is the raw material of the actual enzyme required for starch hydrolysis, α-amylase. Barley malt costs $1.00 per kg and pure barley α-amylase costs $420 per kg (Sigma-Aldrich 1995 catalogue). The detailed composition of this barley malt is shown below:

Table B.3: Composition of Barley Malt

Based on 100 g of barley malt:	
COMPONENT	**AMOUNT**
Protein	10.3 g
Moisture	8.21 g
Ash	1.37 g
Carbohydrates	78.3 g
Fats	1.84 g
Sodium	0.011 g
Vitamin A	19 I.U.
Vitamin C	0.0006 g
Calcium	0.037 g
Iron	0.0047 g

(4) **Calcium Chloride** ($CaCl_2$): Calcium ions are important components of the medium. The α-amylase enzymes are calcium metalloenzymes and are hence, completely unable to function in the absence of calcium ions.